ANNUAL REPORT
OF
PROGRAM ACTIVITIES
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
Fiscal Year 1982

DIVISION OF HEART AND VASCULAR DISEASES
ANNUAL REPORT
October 1, 1981 - September 30, 1982

General Mission:

The Division of Heart and Vascular Diseases plans, conducts and supports a broad program of basic, applied and clinical research, clinical trials, research training of investigators and demonstration and education research directed at the problems of heart and vascular diseases. The ultimate goal is to acquire knowledge of the basic causes of these diseases to enable increasingly effective prevention and reduction of the adverse consequences of these diseases.

Cardiovascular diseases still rank first as causes of death in this country and accounted for an estimated 1,005,000 deaths or 51 percent of all deaths in 1980. From national health interview survey estimates, at least 35 million persons have one or more of the heart and vascular diseases. Additionally, if persons with mild high blood pressure are included, a total population of more than 60 million are affected by these diseases. An economic cost of $80 billion has been estimated for cardiovascular diseases in 1979. Of this amount $30 billion was for direct costs of medical care and $50 billion in lost productivity resulting from illness and premature deaths.

The mission of the Division is to gain the knowledge to reduce the toll from these widespread afflictions which are a major personal and economic burden on the population.

Progress Toward Achieving Objectives

During the reporting period many evidences of progress against the heart and vascular diseases have been documented. These are the products of prior investments in supporting a broad base of productive biomedical scientists in many medical institutions who have been attracted and stimulated to work in this challenging area. Examples of continued progress are:

- A downward trend in the overall death rate from the major cardiovascular diseases has continued at an average of 2.3 percent per year since 1972 or a 22 percent reduction in the past 10 years. The age-adjusted decline in mortality between 1968 and 1978 was 25 percent for coronary heart disease, a reduction of 37 percent for stroke mortality, a 53 percent reduction for hypertensive diseases mortality and a 38 percent reduction in rheumatic heart disease mortality. There was an upturn in 1980 coincident with two severe influenza epidemics, however in 1981 the provisional unadjusted death rates from the major cardiovascular diseases have shown a 2.2 percent decline. The overall trend therefore continues to be favorable.

- The productivity of fundamental and clinical investigations in heart and vascular diseases is indicated by the continued growth of scientific reports presented at scientific meetings throughout the world. Some 4,000 abstracts were submitted and 1,263 abstracts accepted for the 54th Scientific Sessions of the American Heart Association in November 1981. Division supported grantees are the

predominant contributors of papers presenting new research findings at this meeting. Such meetings provide interchange among basic research scientists, clinical researchers, established investigators and young new investigators and are especially valuable in stimulating rapid dissemination of research findings. Similar growth of publications has led to creation of new journals devoted only to cardiovascular research reports.

Highlights of Research

Highlights of Atherosclerosis Research

- Synthesis and Secretion of Lipoprotein Lipase by Macrophages

  It has been demonstrated that macrophages (human, rabbit, mouse) secrete an enzyme with all the characteristics of post-heparin lipoprotein lipase.

  This new finding introduces a new mechanism whereby macrophages may contribute to atherogenesis. By secreting lipoprotein lipase, the macrophage can degrade triglyceride-rich lipoproteins that get into the subendothelial space (through the endothelium or after endothelial injury) and the cell can then take up the products of the action of lipoprotein lipase (lower glycerides, free fatty acids, lysolecithin). Furthermore, the action of lipoprotein lipase on chylomicrons and/or very low density lipoproteins may generate a smaller lipoprotein for which the macrophage has receptor activity. The preliminary modification of the lipoprotein by lipoprotein lipase may generate a "remnant" that is then taken up avidly as a particle. (Arteriosclerosis SCOR: University of California @ San Diego, - Dr. Daniel Steinberg, Director)

- Endothelial Cell Modification of Native low density lipoprotein and Uptake of Modified LDL by Macrophages

  Macrophages and arterial smooth muscle cells are the putative precursors of the lipid-laden "foam cells" in atherosclerotic plaques. The transition of smooth muscle cell to foam cell presumably reflects lipid accumulation following the binding and uptake of native LDL by specific high affinity receptors on the cell surface. Because macrophages lack the high affinity receptor and only take up native LDL slowly, alternative mechanisms have been sought to explain the macrophage to foam cell transformation in atherogenesis.

  Macrophages have been shown to contain a specific high affinity binding site for chemically-modified LDL. A possible physiological role for this receptor has now been indicated.

  It has been shown that native LDL incubated with endothelial cells in culture undergoes a modification characterized by an increase in density and electrophoretic mobility. The endothelial cell-modified LDL is taken up and degraded by macrophages 3 to 4 times more rapidly than native LDL, and its uptake and degradation is competitive with that of chemically-modified LDL. (Arteriosclerosis SCOR: University of California @ San Diego, - Dr. Daniel Steinberg, Director)

- Lower All-Cause Mortality in Estrogen Users.

  The influence of exogenous estrogen use and hysterectomy status on all cause mortality (72 deaths) was examined for 2,269 white women, 40 to 69 years old, who had been followed for an average of 5.6 years in the Lipid Research Clinics Follow-up Study. Compared to estrogen non-users, the relative risk of death in estrogen users was 0.54 in intact women, 0.34 in hysterectomized women, and 0.12 in bilaterally oophorectomized women. The relative risk of death in estrogen users, irrespective of hysterectomy status, was 0.37 times that in non-users (3.4/1000 vs 9.3/1000). The significant protective effect of estrogen use persisted after multivariate adjustment for confounding factors. Hysterectomy status alone was not a significant predictor of total mortality. Multivariate analyses suggest that some of the apparent protective effect of estrogen use can be explained by increased levels of high density lipoprotein cholesterol.

  The consistency of the LRC results provides evidence for an apparent protective effect of exogenous estrogen use. However, since the physiologic effects of estrogens are varied and complex, and other mechanisms for risk reduction may be operating, more detailed investigations are warranted. (North American Lipid Research Clinics)

- Psychosocial Factors and Coronary Artery Arteriosclerosis

  A relationship of psychosocial factors (behavior and environment) to coronary artery disease has been demonstrated in studies in which monkeys determined to be dominant or submissive in behavioral traits were maintained on an atherogenic diet in colonies representing a socially stable or unstable environment.

  Dominant animals in the stable environment had less coronary artery arteriosclerosis while dominance in the unstable situation resulted in more disease. Presumably, the assumption of dominance in the stable environment imposes less stress on the individual animal, whereas the continuing challenge for dominance in the unstable environment exacerbates stress and disease.

  The findings represent the first demonstration of a direct anatomic relationship between the extent of coronary artery disease and psychosocial factors. Arteriosclerosis SCOR: Bowman Gray School of Medicine - Dr. Thomas Clarkson, Director

- LDL Receptor Defects in Familial Hypercholesterolemia.

  Familial hypercholesterolemia, characterized by elevation of total cholesterol, low density lipoprotein-cholesterol, xanthomatosis, and early development of cardiovascular disease, is one of the most common simply inherited disorders in man. Investigators have found that it results from a defect in the specific cell surface LDL receptor, which normally controls LDL catabolism and intracellular cholesterol synthesis. Studies of skin fibroblasts obtained from patients diagnosed clinically as homozygous for the FHC gene, and their first-degree relatives, suggest the existence of at least three mutant alleles at the LDL receptor locus. As a result of this work, it is now quite obvious that familial

hypercholesterolemic patients who are virtually indistinguishable clinically can be geneticaly quite different, have different risk of disease and life expectancies, as well as have different responses to therapy. In addition, this work is providing important insights on pharmacological and dietary influences on LDL levels, and thus it is highly relevant to our understanding and control of atherogenesis. (Drs. Brown and Goldstein, University of Texas - Southwestern Medical School)

- Abnormality of Apolipoprotein E and Familial Dysbetalipoproteinemia

  Recent studies have considerably advanced our understanding of the specific defect underlying familial dysbetalipoproteinemia (also called Type III hyperlipoproteinemia), a disorder that leads to premature atherosclerosis and coronary heart disease. Researchers have characterized familial dysbetalipoproteinemia as a quantitative and qualitative abnormality of apolipoprotein E, which plays a major role in the binding of lipoproteins to cell surface receptors. The synthesis of this apolipoprotein has been found to be under the control of three alleles (E^2, E^3, and E^4) at a single genetic locus. Homozygosity for the E^2 allele gives rise to an apoE phenotype that predisposes the subject to dysbetalipoproteinemia. The heterogeneity of the three major apoE isoforms is due to structural differences between them. Based on these advances in biochemical elucidation of apoE, further studies are now being undertaken to assess the significance of the various apoE isoform phenotypes for coronary heart disease in the general population. (Drs. Mahley and Weisgraber, Gladstone Foundation for Cardiovascular Research; Drs. Breslow and Zannis, Harvard Medical School; Dr. Utermann, University of Marburg, West Germany; and Drs. Brewer and Schaefer, NHLBI).

- Structure and Function of High Density Lipoproteins (HDL.

  Prostacylin (PGI_2) is a vasoactive prostaglandin synthesized by vascular endothelial and smooth muscle cells. This P.I. has reported data that HDL stimulates PGI_2 synthesis in cultured arterial endothelial cells. This effort could potentially contribute to the known inverse correlation between HDL concentration and the risk of coronary heart disease. The mechanism for increased PGI_2 synthesis could be the supplying to endothelial cells of arachidonic acid by the HDL. (Dr. Alan Tall, Columbia University)

- Endothelial Injury and Regeneration.

  The proliferation of smooth muscle cells (SMC) in blood vessels after endothelial injury is a key step in the pathogenesis of atherosclerosis, and is also thought to be a major cause of failure of vascular surgeries. The observation of Dr. Karnovsky that non-anticoagulant heparin prevents SMC proliferation may well find clinical applicability in terms of preventing serious consequences of SMC hyperplasia following vascular surgery. The identification by this investigator of antiproliferative but not anticoagulant heparins, such as the N-desulfated molecule, may be very promising in this regard. (Dr. Morris Karnovsky, Harvard Medical School)

- Effect of Salmon Oil Diet on Plasma Lipids and Lipoproteins.

The lower plasma lipid levels and lower incidence of atherosclerotic diseases in Greenland Eskimos suggested that the unusual fatty acids present in their diet of seal and fish may be anti-atherogenic. These fatty acids are of the omega-3 fatty acid family. A salmon oil diet containing high levels of these unique fatty acids to a control diet high in saturated fat and to a vegetable oil diet high in linoleic acid (C18:2) was compared. In four weeks the salmon oil diet reduced plasma cholesterol levels from 188 to 162 mg/dl and triglyceride levels from 77 to 48 mg/dl respectively, with corresponding changes in LDL and VLDL. HDL cholesterol levels did not change. The vegetable oil diet caused similar decreases in cholesterol levels but did not lower triglyceride levels. These unusual omega-3 fatty acids comprised up to 30% of the total fatty acids in each plasma lipid class after the salmon diet. Fish oils contain fatty acids which may be metabolically unique and potentially useful in the control of both hypercholesterolemia and hypertriglyceridemia. (Dr. William Connor, University of Oregon School of Medicine)

- Microcirculation in Patients with Diabetes Mellitus.

Quantitation of the types of microvascular changes that accompany diabetes in young individuals with insulin dependent disease has shown that two diametrically opposite alterations occur. At the capillary level the total length of the exchange vessels is increased very early but then falls off until in 30-40 year old subjects capillary density is less than that of non-diabetics. In contrast, the compliance vessels (venules) become increasingly longer, more prominent and tortuous - a phenomenon encountered normally only in individuals 50 years of age or older. Such studies of the microvascular network are significant in that they provide a hitherto unavailable insight into the secondary manifestations of the disease process, as well as a yardstick of the effectiveness of the treatment of the diabetes (Dr. Benjamin Zweifach, University of California @ San Diego)

- Surgical and Metabolic Aspects of Venous Grafts

When synthetic or venous autografts are inserted into the arterial circulation, platelet adherence to the injured area is stimulated; subsequently there is proliferation of smooth muscle cells, and intimal thickening is the universal response to grafting which is seen. This early intimal thickening appears to be self-limiting in time and is not related to hyperlipidemia. However, in a subhuman primate model, Dr. Hagen has observed that hyperlipidemic animals develop atherosclerosis after six to fifteen months, and the areas of intimal thickening are particularly susceptible. Medication with aspirin and dipyridamole reduces intimal thickening in vein-grafted monkeys (M. fasicularis), both in the graft per se and in arteries adjacent to synthetic vascular prostheses. This finding is of potential clinical importance since patency was 100% in this study. It is suggested that the effect of aspirin is on platelets and thrombogenic events, while the major effect of dipyridamole, as shown by thymidine uptake studies, may be on smooth muscle cells by interfering with cellular transport systems. (Dr. Otto Hagen, Duke University Medical Center)

Highlights of Coronary Heart Disease Branch

- The Beta-Blocker Heart Attack Trial (BHAT)

  The Beta-blocker Heart Attack Trial (BHAT), originally scheduled to conclude in June 1982, was terminated in October 1981 because of the substantial and significant decrease in mortality seen in the propranolol treated patients. Results (published in the November 6, 1981, and March 26, 1982, issues of JAMA) indicated that patients receiving propranolol experienced a 26% lower mortality from all causes than did the placebo control. Total mortality during the average 25-month follow-up period was 7.2% in the propranolol group and 9.8% in the placebo group. Arteriosclerotic heart disease mortality was 6.2% in the propranolol group and 8.5% in the placebo group. Sudden cardiac death was 3.3% among the propranolol patients and 4.6% among the placebo patients.

- The Multiple Risk Factor Intervention Trial (MRFIT)

  The intervention phase of the Multiple Risk Factor Intervention Trial (MRFIT) was completed on February 28, 1982. Endpoint results will be announced at a press conference scheduled for September 16, 1982, and a publication is planned for the September 24 issue of JAMA. This major primary prevention clinical trial was undertaken to determine whether the reduction of serum cholesterol, elevated blood pressure and cessation of cigarette smoking over a 6 year period would result in a lower mortality from coronary heart disease.

- Coronary Artery Surgery Study

  The Coronary Artery Surgery Study (CASS) has entered its final year of followup. The Trial is scheduled to close in May 1983. The 25,179 patients enrolled in the Registry and the 780 patients randomly assigned to medical or surgical treatment will have been followed for an average of 6 years, a minimum of 4 years, and a maximum of 8 years at that time. The enormous data base is being analyzed, and publication of trial results is anticipated in the Fall of 1983. The Coordinating Center will be supported for 3 years beyond 1983 to permit adequate analysis and publication of results.

- Percutaneous Transluminal Coronary Angioplasty Registry

  The Registry has collected baseline and acute outcome data on over 3,000 patients treated with this procedure in the United States and Europe. Data Coordinators have been retained at 12 of the most active sites, and long term followup on all patients from these sites will be completed by October 1982. Analysis and publication are proceeding.

  It appears that this procedure may be useful in approximately 10% of patients (single vessel disease with approachable lesions) undergoing coronary arteriography because of angina. Success rates vary between 60% and 80% depending upon physician experience. Mortality rates are less than 1%. New catheter designs which promise to simplify the technical

aspects of the procedure are being introduced into clinical practice. Investigators are cautiously applying this technique to patients with multivessel disease under various experimental protocols.

- Non-Invasive visualization of atherosclerotic plaques

Improvements continue to be made in intravenous subtraction angiography techniques for imaging arterial lesions. One contractor has demonstrated that energy subtraction techniques when combined with the more traditional temporal subtraction techniques yields improved image quality by minimizing motion antifacts. Two other contractors have performed experiments in dogs in which coronary arteries have been visualized with mixed success.

Seven contractors have been engaged in a program to assess the capabilities of ultrasound B-scan systems to detect and quantify lesions in arteries. Common collaborative protocols have been completed for the human studies, including history, B-scan studies, angiography and anatomic measurements from surgical specimens. Pilot studies are underway.

- Psychosocial Factors in Coronary Heart Disease

Three papers have been completed and two public presentations have been given that relate the ten-year incidence of coronary heart disease in men and women to characteristics of their spouses. Data from the Framingham Heart Study has demonstrated that the social status of wives as measured by education and occupation is related to the occurrence of CHD in husbands. The spouse data also demonstrated that the differential rate of CHD between Type A and Type B men was present only in situations where the wives' characteristics might be deemed stressful. These results were apparent regardless of the husbands' social status and standard coronary risk factors.

Social status characteristics of husbands were not related to the occurrence of CHD in wives. However, certain psychological characteristics of husbands are associated with the ten-year incidence of CHD in women.

- Biobehavioral Research in Coronary Heart Disease

Previous biobehavioral research has shown that defined behavior patterns, Type A, are significantly correlated to coronary heart disease morbidity and mortality. Several NHLBI research grants are currently attempting to identify the biological mechanisms whereby these behavioral patterns are linked to cardiovascular disease. Enhanced and distinct patterns of sympathetic nervous system activity and neuroendocrine responsivity have been identified (Williams, HL22740) and the possibility of a genetic basis for some elements of coronary prone behavior is also being studied (Dembroski, HL22809). Additionally, laboratory stress tests have been used to identify the differences in coping behavior related to coronary prone behavior and to provide a basis for the prediction of physiological reactivity and consequent coronary disease for patterned reactions to environmental stressors (Glass, HL22514).

Highlights of Hypertension Research

- Reduction of Stroke Mortality by Antihypertensive Therapy

  In a major publication from the Hypertension Detection and Follow-up Program this year, the five-year reported stroke incidence rate was significantly lower in Stepped Care than in Referred Care (1.9% vs. 2.9%). Reductions for Stepped Care were experienced for all race-sex groups, DBP strata and age groups in the study (JAMA, 247(5), 633-638, February 5, 1982). In another publication this year, it was noted that with the use of a life table regression method of analysis to adjust for actual time of reserpine exposure, race, sex, and medication status at entry and comparing those who took reserpine with those who did not, a risk ratio of 1.28 with a confidence interval of 0.58 to 2.80 was calculated. Thus, a statistically significant association was not demonstrated for reserpine use and breast cancer over the five years of the study.

- Human Renin Purified

  Human renal renin has been further purified by sequential chromatography into two major components, one of which has a smaller molecular weight (apparently a break-down product of the intact human renin). This finding points to the marked instability of this enzyme under certain conditions and suggests that the actual molecular size is larger than reported previously.

  This purification is important because

  1) Renin is the key enzyme in the production of angiotensin II, the major hypertension-causing vasopressor.

  2) Antibodies to renin have already been shown to lower blood pressure in animals. Pure human renin will make possible the development of specific human antibody for a parallel study in humans.

- Effect of potassium citrate on salt hypertension in rats

  The salt hypertension rat model (Dahl-S rat) develops nephrosclerosis and nephron loss when fed a high salt diet (4% NaCl) for 24 weeks. However, when this model is given a supplement of potassium citrate it is protected against nephron loss even though the blood pressure remains elevated. By contrast, an equivalent supplement of potassium chloride neither lowers the blood pressure nor protects against nephron loss. Apparently, the citrate (in combination with potassium) provides the protection.

- Hypertension and Obesity in Humans

  Hemodynamic and volume studies on moderately obese hypertensives, non-obese hypertensives and normals were conducted at the Cleveland Clinic over a five year period. After studying a total of 134 subjects the results indicated that hypertension in the obese cannot be defined by a

single functional abnormality such as high cardiac output or expanded blood volume, nor can it be distinguished from hypertension in the non-obese by standard hemodynamic or humoral measures.

Thus, the question, "Why does weight loss reduce blood pressure?", still remains to be answered.

- Nutrients with Blood Pressure Effects

Nutrients ingested as food can act like drugs. Three amino acids, choline, tyrosine, and tryptophan, are precursors of neurotransmitters, and when they are ingested they give rise to nervous system effects. In turn these may affect the cardiovascular system. Studies done at MIT show that tyrosine in the diet can either increase the synthesis of norepinephrine or leave it unchanged, depending on the firing frequency of particular neurons. When tyrosine is administered to animals with normal blood pressure, there is no consistent effect. However, when it is given to hypertensive rats, it lowers their blood pressure; given to hypotensive animals (in shock, for example), it raises their pressure to near normal levels. The mechanism of action seems to be that the norepinephrine associated with tyrosine ingestion either inhibits the activity of the peripheral sympathetic neurons (in the case of hypertensive animals), or suppresses the inhibitory norepinephrine-releasing neurons of the brain stem (in the case of animals in shock). This research was conducted in collaboration with Boston University Hypertension SCOR.

- Behavioral Defects in Hypertension

In the area of hypertension, the demonstration that mild, untreated essential hypertensives have a significant defect in their behavior as evidenced by data from sensory-perceptive, cognitive and psychomotor testing has been most interesting (Shapiro, HL20724). These differences would not interfere with normal everyday activities but represent a behavioral equivalent of the cardiovascular defects in hypertensives which appear during stress testing. Quite striking is the evidence that the defects are more marked in the female hypertensive than in the male. These defects may be reversible by treatment of hypertension with effective lowering of the blood pressure, however, certain new defects may become apparent depending upon the type of pharmacologic therapy employed.

- Factors Related to 10 Year Incidence of Stroke in Japanese-Americans

As part of an ongoing longitudinal study among Japanese men in Hawaii, 8006 men of Japanese ancestry living on the island of Oahu and aged 45 to 68 at entry examination have been followed by reexamination and surveillance. One hundred and eleven men were found to have evidence of prior stroke at the time of the initial examination. During a ten-year follow-up of the remaining 7895 men, 154 developed thrombo-embolic stroke, 65 intra-cranial hemorrhage, and 19 developed stroke of unknown type. There were 79 deaths attributed to stroke. The principal risk factors for thrombo-embolic stroke were elevated blood pressure, glucose intolerance, age, electrocardiographic evidence of left ventricular hypertrophy or

strain, and cigarette smoking. Attributes associated with increased risk of intra-cranial hemorrhage were elevated blood pressure, electrocardiographic evidence of left ventricular hypertrophy or strain, cigarette smoking, and alcohol intake.

Analysis of the relation between dietary factors and the occurrence of stroke revealed an inverse relation between total fat intake and the incidence of total stroke and of thrombo-embolic stroke, but no relation between fat intake and the incidence of intra-cranial hemorrhage. Fat intake was also inversely related to stroke mortality. When fat as a percent of calories was used in the analysis, the inverse relations persisted. No relation was found between salt intake and the incidence of or mortality from stroke.

Highlights of Studies of the Heart

- Visualization of Fine Structure by Sonication

A new method for use with scanning electronmicroscopy which involves overfixation of biological tissue with osmic acid and subsequent sonication has been described. The method has particular application in the study of vascular beds and promises to be extremely useful in the microdissection of biological tissue. (Frank N. Low, Principal Investigator, Louisiana State University, HL 28365)

- Neural Regulation of Subsidiary Atrial Pacemakers

Subsidiary atrial pacemakers develop in the canine heart after surgical excision of the anatomical sinoatrial node. The investigators propose a major new concept which views the sinoatrial node as an extensive distribution of pacemaker cells along the course of the crista terminalis. The subsidiary pacemakers and not the atrioventricular node assume the dominant pacemaker role in the absence of the sinoatrial node. (Walter C. Randall, Principal Investigator, Loyola University, HL 28205)

- Arachidonic Metabolites and Vascular Reactivity

It has been found that urinary immunoreactive thromboxane B_2 (i-TXB_2) is markedly elevated several days prior to renal allograft rejection. It is suggested that urinary i-TXB_2 may be a simple and non-invasive test to facilitate the early diagnosis of venous thrombosis. (Peter W. Ramwell, Principal Investigator, Georgetown University, HL 18718)

- Degradation of Cardiac Myofibrils and Mitochondria

A technique has been developed for both the separation and quantitation of the individual classes of myosin heavy chains. Moreover, monoclonal antibodies have been classified which are specific for the high or low ATPase variants of the heavy chains, irrespective of the animal species. These techniques will make possible both turnover studies and comparative studies which will provide information concerning the physiological meaning of myosin polymorphism and the mechanisms of cardiac hypertrophy. (Radovan H. Zak, Principal Investigator, University of Chicago, HL 16637)

Highlights of Therapy of Severe Cardiac Diseases

- Heart-Lung Transplantation

  Investigators at Stanford University have performed combined heart-lung transplantation in six patients. There are four survivors; the first successful combined transplant in the United States was performed at Stanford in March 1981. A new effective immunosuppressant drug, Cyclosporin A, has permitted improved management of graft rejection.

- Implantable Electrically Powered Left Ventricular Assist Systems

  In FY '80 NHLBI issued an RFP for the development of implantable, integrated, electrically powered left heart assist systems. The major goal of this program is to develop and evaluate implantable electromechanically actuated left heart assist systems with long term durability and reliability (two years or greater) and tether-free operation. Five contract awards were made in August 1980. Efforts are being directed toward the development of systems which will be suitable for pre-clinical validation at the end of the four year contract period. Current accomplishments include the miniaturization of key components and the initiation of characterization studies.

  Impressive accomplishments have been made in the life testing of these systems. Several energy converters have achieved the equivalent of over two years of life on accellerated life tests and one converter has achieved 132 million cycles (3.14 years) without failure. Blood pump bladders have achieved the equivalent of five years of life without failure. Progress has also continued on the development of volume compensation for pulsatile blood pumps. A current thoracically implanted, lenticular shaped, textured compliance chamber is approaching two years of operation in a calf without significant tissue encapsulation.

Significant Workshops, Task Forces and Meetings

The DHVD has undertaken a Ten-year Review and update of the National Program Plan during the reporting period. Heart and Vascular Disease Task Groups have reviewed the progress and accomplishments in ten of the major areas of Division responsibility for the period 1972-1982. Recommendations for Research initiatives and the development of new researchers over the period 1982-1987 have also been submitted. The reports of the Task Groups are scheduled for completion in November 1982 and should provide a valuable planning resource for the Division and for NHLBI.

Major Problem Areas

A. PERSONNEL

Personnel needs of the Division require recruitment of scientific personnel for key posts that need to be filled to maintain high quality management of Division research programs.

B. SPACE

Space needs in the Federal Building continue to remain unsolved although efforts of NIH Space Management and GSA are attempting to correct long-standing problems. These include:

- Fascia

  There is still no information as to when the removal and installation of the fascia on the outside of the building will begin; the materials are still on order. This situation is extremely dangerous and someone could be struck by a piece of falling fascia.

- Painting

  We were informed that 16 offices would be painted in the building, but NIH Space Management would not accept that and informed GSA that all office space must be painted. To date, there is no further word on if or when this will be accomplished.

- Replacement of Venetian Blinds

  There are many blinds that have tapes that are badly worn, cords that are broken, and blinds that cannot be adjusted. We understand that a service call is all that is needed to get these repaired but we have, so far, received no response or service to our repeated calls.

- Office Lighting

  We have requested replacement of all surface mounted flourescent ceiling fixtures with the new flush mounted drop-ins. All surface mounted fixtures either have broken or missing diffusers and all are so stained that instead of getting white light, it is a soft yellow light.

- Building Supplies

  The delivery of paper and cleaning supplies to the Federal Building for the washrooms is inadequate.

- Fresh Air

  Due to the multiple renovations that have been made in the Federal Building over the years, the flow of fresh air to certain areas has been curtailed; this becomes especially apparent between 4:00-4:30 PM when the fans are turned off.

C. RESEARCH TRAINING

Research training and development of promising new scientists continue to be impeded by uncertainties of funding levels. Shortage of investigators in specific areas has been identified and such shortages are unlikely to be corrected under the "stabilization" levels in place. Particular

categories of shortage of investigators are: clinical cardiovascular investigators, pediatric cardiology investigators, medical nutrition scientists with cardiovascular training, epidemiologists, biostatisticians, clinical investigators for clinical trials, cardiomyopathy research, peripheral vascular disease research and biomedical engineers.

D. BASIC SCIENCE RESEARCH

Lack of growth of research funds in the setting of an increased number of meritorious research applicants recommended for approval has created a dilemma for the Division. For the first time the prospect is emerging of a strong biomedical resource of scientists who will face termination of support for further research because competitive ranking of merit of their research applications is not within fundable priorities. Increasing cost of uncontrollable areas as indirect costs reduce the funds available for direct support of the research that peer scientists have recommended for funding. The dissolution of large research groups and research applications for new individual small projects from investigators previously funded within Program Projects are being seen. The increasing evidence of a need for interdisciplinary research in the more complex areas of basic science raises concern that the support for such research will become increasingly difficult.

DIVISION OF LUNG DISEASES

ANNUAL REPORT

October 1, 1981 through September 30, 1982

I. MISSION

The broad program goals of the Division of Lung Diseases are more effective diagnosis, treatment and, ultimately, prevention of pulmonary diseases. To these ends, the Division emphasizes seven program areas: structure and function of the lung (including lung development); chronic obstructive lung diseases (emphysema, chronic bronchitis, asthma); pediatric pulmonary disease (neonatal respiratory distress syndrome, cystic fibrosis, bronchiolitis); fibrotic and immunologic interstitial lung diseases; pulmonary vascular diseases (pulmonary hypertension, pulmonary edema); respiratory failure; and prevention, control and education. These programs are implemented through four branches: Structure and Function; Airways Diseases; Interstitial Lung Diseases; Prevention, Education and Manpower.

In addition to supporting investigator-initiated regular research, program project and training grants, research career awards, and new investigator research grants, the Division has stimulated research or training in specific areas through goal-oriented initiatives. Grants for specialized centers of research (SCORS) support interdisciplinary investigations with a clinical focus. A grant for a lung research and demonstration center supports demonstration and education projects to prevent and control pulmonary diseases. To meet the need for additional research and clinical manpower, the Division initiated the Pulmonary Academic Award and Pulmonary Faculty Training programs. Research contracts are used to foster targeted pulmonary research and development projects.

II. PROGRESS TOWARD OBJECTIVES

The investigator-initiated research grant continues to be the major source of innovative fundamental research. It contributes not only to our understanding of normal lung function and the processes involved in respiratory disorders, but also leads to improvements in the diagnosis and management of pulmonary diseases. From time to time, the Division supplements these programs by issuing requests for applications (RFAs) or for contract proposals (RFPs) to encourage exploration of problems not being addressed, or being insufficiently addressed, by investigator-initiated grants. The areas where stimulation is needed are identified through working groups, workshops, and task forces, as well as by

the Pulmonary Diseases Advisory Committee and the Division's professional staff. One program area in the Division's National Plan continues to depend almost entirely on special initiatives; namely, the Prevention and Control program.

The working group on the Natural History of PiZ Emphysema has collected and analyzed retrospective data on 300 American subjects with alpha-1-antitrypsin deficiency. This is the largest available database of changes in pulmonary function over time in PiZ individuals. At their third meeting the group discussed data on 21 selected PiZ subjects and an additional 29 Swedish individuals of the PiZ phenotype. The American and Swedish data are very similar and thus are thought to be representative of a group of PiZ individuals who develop frank airflow limitation. The decline in lung function of these PiZ individuals is greater than the decline in function found in other COPD patients, suggesting that PiZ subjects who have significant airflow obstruction go through a phase of their disease during which FEV_1 declines at a rapid rate. If a trial of alpha-1-antitrypsin therapy becomes feasible in the future, the effects of treatment might be demonstrable in such a group of patients. A paper describing the findings of the workshop is being prepared.

The Division sponsored a workshop on Comparative Biology of the Lung. The purposes of the workshop were to provide reliable morphologic data on the developing and mature respiratory system in animal species commonly used for research, to compare them to man, and to attempt to establish structure-function relationships. Proceedings of the workshop will be published in the American Review of Respiratory Disease and will contain an easily accessible index on morphological features. The report will also identify gaps in our knowledge and discuss the limitations inherent in extrapolating data from animals to man.

In recent years there has been a rapid accumulation of data on hormones, neurotransmitters and "growth factors" which may influence lung development. A workshop on factors which regulate Fetal Lung Development, supported by a conference grant and involving investigators from a variety of basic and clinical disciplines, reviewed these data and provided recommendations and guidelines for future investigation. Proceedings of the workshop will be published in the Journal of Pediatrics. A workshop on The Endocrine Lung in Health and Disease, also supported by a conference grant, brought together investigators from the United States and abroad to focus upon the pathophysiology of the pulmonary endocrine cell, humoral substances produced by other cells in the lung, and structural, functional, and developmental relationships between the pulmonary endocrine cell and the neoplasms to which they may give rise. The proceedings will be published in book form.

A pediatric SCOR coordination workshop was conducted on High Frequency Ventilation (HFV) in Immature Infants. The workshop participants discussed in detail the physiological principles of gas exchange during

HFV, the state-of-the art of this technique in human and animal newborns, and its possible risks and benefits in this age group. It was concluded that the mechanisms of gas exchange during HFV, and the risks and benefits of this technique need to be elucidated further before a systematic evaluation of HFV can take place in a clinical setting. The summary and recommendations of the workshop will be published in Journal of Pediatrics.

Three workshops sponsored by the Division, Arachidonic Acid Metabolites and the Pulmonary Circulation, The Fibroblast in Interstitial Lung Diseases, and Exercise Testing for the Dyspneic Patient will be held in September, 1982.

The Patient Registry for Primary Pulmonary Hypertension is now in full operation. Thirty-five clinical centers, which include over 60 hospitals, are participating in the registry. The patient reporting forms, patient protocol and manual of operations have been distributed to all the participating clinical centers. One-day training sessions, under the direction of the steering committee, data center personnel and DLD staff, were held in New York, Chicago, and Denver in early summer. The purpose of the meetings was to instruct the personnel from each clinical center on the use of the patient reporting forms.

The Intermittent Positive Pressure Breathing (IPPB) Clinical Trial is continuing to gather data on 986 patients. The study will compare the relative effects of long term IPPB and compressor powered nebulizer treatment when used as an adjunct in the comprehensive care of ambulatory patients with chronic obstructive pulmonary disease (COPD). The effects to be measured include pulmonary function, hospitalization, quality of life, exercise performance, and rates of attrition. The safety of the two devices will be measured by assessing mortality and incidence of infections. Data collection in the trial will continue until April of 1983, allowing for a 30-36 month follow-up on all patients.

During the past year the major short-term outcomes of the collaborative, double-blind clinical trial to assess benefits and risks of antenatal steroid therapy have been published in the American Journal of Obstetrics and Gynecology. An overall significant reduction in incidence of neonatal respiratory distress syndrome (from 18.0% to 12.6%) was observed when up to 20 mg of dexamethasone was administered to the mothers prior to delivery. Surprisingly, the effect of dexamethasone was modified by sex and race of the infant. The incidence of RDS in the female infants was reduced by more than 75% (from 18.0% to 4.8%) whereas no reduction was observed in males. In addition, Caucasians showed little benefit from treatment compared to non-Caucasians. No adverse effects in terms of fetal or neonatal mortality, neonatal infection and neurologic abnormalities, or maternal infection were attributable to the dexamethasone treatment. The study, will continue through August 1983 and results of long-term follow-up are needed before final assessment of risks versus benefits can be made.

In the past four years the NHLBI has supported three contracts to develop model self-management programs for children with asthma and their parents. This year, the NHLBI and the NIAID have developed a project to evaluate these programs along with other behavioral programs for education of the asthmatic child. The evaluation project is being funded with one percent evaluation set-aside funds from the Department of Health and Human Services. Experts in behavioral science and health education are writing an evaluation report focusing on many aspects of asthma self-management. Conclusions and recommendations for future work will be developed at a workshop in February 1983.

The Pulmonary Academic Award (PAA) evaluation project, conducted under contract to the Association of American Medical Colleges, is now complete and a final report is available. The study compared 14 schools that received PAAs in the early years of the program with a similar group of medical schools that did not have PAA support. While the study did not uncover any effect of the program on student interest or career choice in pulmonary medicine, it did find significant effects on the pulmonary teaching programs at the PAA schools. Specifically, ratings of the pulmonary curriculum, clinical sciences teaching, and clinical faculty quality improved measurably in the schools that held PAAs. Further, student knowledge of pulmonary medicine, as measured by performance on parts I and II of the National Board of Medical Examiners tests, increased significantly for schools that held PAAs. While the results of this pilot evaluation study must be interpreted cautiously, they suggest that the Pulmonary Academic Award has been successful in meeting its goal of providing a better pulmonary education to undergraduate medical students.

The Clinical Investigator Award Program continues to be a successful means of attracting physicians to pulmonary research.

As a special initiative to encourage studies on the processes by which the lung initiates and develops immune reactions prior to or during pulmonary fibrosis, the Division issued an RFA for studies of Immune Responses in the Lung Underlying Interstitial Pulmonary Fibrosis. Twenty applications were approved (out of twenty-six reviewed) by a special study section, and following approval by the NHLBI Advisory Council, the Division initiated six awards in July 1982. These studies should add to our understanding of immunologic processes in the lung, and thus should lead to better understanding of the pathogenesis of these diseases as well as improved diagnosis and treatment.

Another special initiative (RFA) invited grant applications on the Structural and Functional Changes Associated with Respiratory Muscle Fatigue. The goal of this program is to encourage collaboration of researchers in different disciplines to elucidate the physiologic, morphologic, biochemical, and neurologic aspects of respiratory muscle fatigue. Although fatigue of respiratory muscles has long been thought to contribute to the morbidity associated with a number of respiratory problems, the exact causes of muscle fatigue have not been explored. The Division hopes to initiate these awards in the fall of 1982.

An RFA for the Pulmonary SCOR Program in Adult Respiratory Failure was

issued in December 1981 inviting new and renewal applications. Thirteen letters of intent were received. The applications are due September 15, 1982. They will be reviewed at the May 1983 Council meeting and funding is scheduled to begin December 1, 1983.

III. HIGHLIGHTS OF RECENT SCIENTIFIC ADVANCES

This section identifies some important advances reported by investigators within the past 2 years. Because of the dimensions of the Division's research program and the breadth of emphasis of the investigations it supports, this summary fails to do justice to the many investigators whose work cannot be represented in this brief account. Some of their accomplishments have been covered in prior reports, others will be presented in the future.

Pulmonary embolism continues to be a major medical problem in this country. It is difficult to diagnose and is often fatal, particularly in patients who have other diseases. In animal studies, acute pulmonary hypertension and hypoxemia accompanying pulmonary embolism have been shown to be dependent in part on the synthesis and release of vasoconstrictor prostaglandins. Prostaglandin levels were studied in 22 patients with presumptive diagnosis of pulmonary embolism. In 10 of the 22 patients, pulmonary embolism was confirmed by angiography and ventilation/perfusion scans. Those with an initial presumptive diagnosis of pulmonary embolism that were not confirmed responded to treatment for other diseases. It was found that thromboxane levels were significantly elevated in the patients in whom the diagnosis of pulmonary embolism was confirmed as compared to those in whom it was not. Levels of prostacyclin, another prostaglandin, remained unchanged in all patients. Since thromboxane is a bronchoconstrictor and pulmonary vasoconstrictor, thromboxane inhibitors may be useful in reducing hypoxemia as well as in reducing prostaglandin-mediated pulmonary hypertension in pulmonary embolism patients. Also, the level of thromboxane may be useful as a diagnostic test in patients suspected of having pulmonary embolism.

Emphysema is a crippling and fatal lung disease that is characterized by a breakdown of the lung's internal structure. While clinicians can infer, from pulmonary function tests, that such a breakdown is occuring, a definitive diagnosis cannot be made without a microscopic examination of lung tissue, done at autopsy. Due to the limitations of current microscopic techniques, many questions about both normal and pathophysiological lung structure and function remain unanswered. A group of investigators are exploring the possibility of using a laser light technique to gain information about lung structure. They have found that when the rays of a laser are aimed at the surface of an excised lung, the pattern and intensity of the light reflected carries with it information about the size and orientation of the alveolar walls. This pattern changes in a characteristic way when the lungs are inflated.

It is anticipated that quantitative estimates of lung geometry can be derived from the observed light patterns. This new technique has the potential to non-destructively explore the structure of both normal and diseased lung, although the technique will have to be developed further before use in living subjects is possible.

It is essential in the management of patients in the intensive care unit to be able to measure the level of arterial oxygenation. The monitoring and regulation of blood gases is particularly important in respiratory failure patients receiving oxygen therapy. Currently, the most widely used method for monitoring blood gases requires that a sample of blood be withdrawn from an artery and the measurement done in vitro. This procedure is invasive and does not allow for continuous monitoring. A method has now been developed that permits the noninvasive, continuous monitoring of arterial blood oxygen through the skin. The method is based on the principle that the amount of light reflected at a specified wavelength is determined by the amount of oxygen in the hemoglobin of the blood. Using this approach, a small optical reflectance transducer placed on the finger tip is being used to measure the transcutaneous arterial oxygen saturation. The results correlate very closely with simultaneous measurements made with conventional methods. Such a technique provides a very low-risk, noninvasive means to continuously monitor surgical patients, intensive care unit patients, and neonates with respiratory distress and other cardiopulmonary problems.

Recent evidence from two clinical trials supported by the Division of Lung Diseases have documented the decrements in quality of life of patients with moderate to severe decreases in pulmonary function, compared to healthy controls. Findings from the completed Nocturnal Oxygen Therapy Trial showed that patients with emphysema and chronic bronchitis who are hypoxemic experience impairments in several aspects of life quality. Depression is the predominent emotional disturbance reported; difficulties in home management, sleep, and reduction in social interaction were also noted. Moreover, significant relationships were found between life quality and measures of neuropsychological functioning in that patients who showed the greatest life quality decrements also exhibited the greatest decrements in neuropsychological functioning. In the IPPB Clinical Trial the neuropsychological functioning of mildly hypoxemic COPD patients was also found to be somewhat inferior to controls.

Studies of host factors that predispose individuals to respiratory disorders, and of environmental factors that increase risk of such disorders in exposed persons continue to investigate a variety of issues.

Long term exposure to air pollution has been thought to contribute to the development of airways obstruction, however, documenting the effect of air pollution has been difficult. Data from a cross-sectional study of residents of two areas in Los Angeles County, one with very high ambient concentrations of photochemical oxidants, nitrogen dioxide and sulfates, and the other with low levels of these pollutants, shows significant differences in symptoms and performance on pulmonary function

tests between individuals from the two areas. Residents of the polluted community reported more respiratory symptoms and had significantly diminished pulmonary function. These findings held true for smokers and nonsmokers of both sexes. However, the largest effects of pollution were seen in nonsmokers and the smallest in heavy smokers. The magnitude of the differences in test results suggests that the deleterious effects of smoking and air pollution are additive. The investigators plan to follow these populations to look for changes in lung function that might correlate with pollutant exposure.

Many nonsmokers are passive smokers in that they regularly inhale smoke coming from cigarettes smoked by other people. Since smoking is the most important risk factor yet identified for the development of chronic obstructive lung diseases, investigators are now interested in whether passive smoking might also harm the lungs. Three years ago an epidemiologic study from Boston indicated that an inverse relationship exists between the score a child gets on a test of lung function ($FEF_{25-75\%}$), and the number of smoking parents in the household. The children whose parents smoked were also reported to have a higher incidence of respiratory symptoms and hospital admissions. A similar study has now been completed in an Arizona population. These investigators concluded that passive smoking in the family does not seriously affect indicators of lung disease such as pulmonary function. At present there is much conflicting data on the long term effects of passive smoking and more information will be needed before this issue can settled.

Patients suffering from COPD have been known to benefit greatly from regular exercise. A major problem with these patients, however is that they have high rates of noncompliance with exercise programs. Recent work has confirmed and quantified the effect which exercise has on COPD patients. In a program designed to increase the amount of exercise that COPD patients routinely perform, it was found that the amount of exercise is related to a variety of outcomes, such as quality of life, health status, subjective symptoms, depression, and cognitive functioning. Moreover, the cost of programs designed to increase activity were recently shown to produce substantial gains in health status relative to other programs at the same cost.

Investigations into how basic processes at the cellular level influence the etiology and treatment of pulmonary diseases continue to yield new and useful information. For example, the role of elastase in the adult respiratory distress syndrome (ARDS) has recently been explored. ARDS can occur as a consequence of a variety of conditions, including pneumonia, gastric acid aspiration, sepsis, and trauma. The disease is characterized by marked respiratory distress, impaired lung compliance, and hypoxemia (low oxygen in the blood). This disease is a major health problem in the United States as approximately 150,000 cases occur annually with a mortality rate exceeding 50%. For some time investigators have suspected that an inflammatory process occurs early in the course of the disease. To explore this possibility, bronchoalveolar lavage fluid collected from ARDS patients and from patients with non-inflammatory diseases has been analyzed. The presence of the enzyme, elastase, which is produced by white blood cells and is known to cause the degradation of lung tissue and the activation of inflammatory

mediators, has been identified in the lung fluids from ARDS patients. Inhibitors of elastase have also been identified; however, they exist in an inactive form. These results suggest that in ARDS patients there is an imbalance between elastase and its inhibitor, which leads to lung injury by acting directly on lung cells and connective tissue and indirectly through the activation of the mediators of inflammation. The effects of therapy on the level of elastase and its inhibitors must be assessed in experimental animals before therapeutic intervention in ARDS patients will be possible.

The role of elastase in emphysema in smokers is also being investigated. A major pathological feature of emphysema is the destruction of the walls of the air sacs (alveoli) in the lung. It has been postulated that this damage is caused by a proteolytic enzyme elastase which digests elastin, a structural element of lung. Elastase/anti-elastase imbalance in the lung has been implicated in the induction of emphysema in individuals genetically deficient in elastase inhibitor alpha-1-antitrypsin (AAT). Animal and in vitro studies indicated that the activity of two major elastase inhibitors, AAT and bronchial mucous proteinase inhibitor (BMPi), can be decreased by oxidizing agents such as those found in cigarette smoke. Recent studies conducted in humans have confirmed the decrease in the anti-proteolytic substances in pulmonary fluids of smokers. This may be the initial event leading to emphysema observed in smokers. This work also indicates that smoking may cause a simultaneous increase in proteolytic activity and a decrease in anti-elastolytic activity, thus facilitating the rapid destruction of elastin. These studies also reveal a biochemical link between emphysema caused by a natural AAT deficiency and that produced in smokers.

For sometime it has been known that endotoxin (a substance produced by many microorganisms and causing a variety of biological effects such as fever and shock) in small quantities can protect adult and neonatal animals from acute lung toxicity of high levels of oxygen (O_2). Further studies of this phenomenon have revealed the surprising finding that rats treated with endotoxin during their first week of life develop a protection against O_2 that is apparent 6 to 8 weeks later when the animals have developed into adulthood living in a normal room air environment. This "memory", which manifests itself as a 3-fold increase in survival rate in adulthood when rats are exposed high to O_2, is not seen if the neonates have been exposed only to high O_2 levels without endotoxin or if adults are given endotoxin and then challenged with O_2 6 to 8 weeks later. There is currently no obvious explanation for this remarkable finding. However, the implication that there may be a critical time in lung development at which a "memory" can be installed for later use has provocative and far-reaching consequences.

Cystic Fibrosis (CF) is a genetic disease which presents at birth as a nutritional disorder and develops into a fatal obstructive lung disease. Nearly one in 1,500 Caucasian babies born in the United States is afflicted with CF. The only diagnostic criterion presently available is elevated salt levels in the sweat of patients. A recent study used a newly developed technique for measuring electric potential differences across nasal mucous membranes, and revealed that the potential differences

in CF patients were more than double that of controls. Topical application of amiloride, a drug which selectively inhibits sodium absorption, reduced the values of CF patients to normal levels. The results further suggested that the increased potential differences across the nasal mucous membranes of CF patients may be due to greater rate of transport of sodium. Excessive sodium absorption, among other things, causes excess water absorption (dehydration) from the surface liquid on the airways, resulting in thickening of mucus. Thickened mucus which is difficult to clear from the lungs, and the associated pulmonary infections, are thus for the first time postulated as arising from the same abnormality that causes excessive salt secretion. The observation that topical application of amiloride reverses the sodium ion-related abnormality in CF may potentially provide new approaches to therapy, if problems related to the delivery of amiloride, its side effects, and dosage etc. can be worked out.

The functions of the many lung cell types in normal lung, and their roles in pulmonary diseases also continues to be actively investigated. Because the lung is a complex tissue composed of many different cell types, the isolation, maintenance and/or cultivation of some of these component cell types could provide powerful tools for the definition of their respective physiologic roles. Although this need has been recognized by many investigators, long-term cultivation has been possible only for a relatively small number of cell types from pulmonary tissue. In 1976, the NHLBI initiated a contract program to develop, characterize and bank as many as 50 cell lines of importance to research on pulmonary physiology. A total of 60 lung cell lines have now been made available by the American Type Tissue Collection (ATTC) through this program. Eighteen species including humans, monkeys, and common laboratory animals are represented. The cells were submitted to the ATTC which carefully characterized and reviewed them, and froze them in liquid nitrogen. Cells are distributed to investigators upon request. The program has permitted banking and provision of well-characterized stocks of cultured cells for pulmonary research, which will be available in uniform condition over the long term. Through use of this resource, questions relating to growth, function, and malfunction of the lung may be approached more readily.

A distinct population of granulated cells possessing both endocrine and neural characteristics has been recognized in the respiratory epithelium for over thirty years. Recent work has demonstrated that the secretory granules of these cells may contain biologically active molecules such as serotonin, bombesin, and calcitonin. The functional importance of these cells is undefined, and despite their abundance in the neonatal lung, little is known regarding the effect of acute and chronic neonatal lung disease on these cells. Anatomic and physiologic evidence, however, suggests that these cells may be in an ideal position to exert control on pulmonary vessel and airway tone either at the local level or throughout the entire lung through vascular and neural connections. A recent study demonstrated that the number of pulmonary neuroendocrine cells is changed in lungs of infants who died of two common neonatal pulmonary disorders where alterations in both pulmonary ventilation and perfusion are known to occur: hyaline membrane disease and bronchopulmonary dysplasia. This study demonstrates that a specific pulmonary cell type containing bioac-

tive molecules undergoes marked changes during acute and chronic neonatal lung disease. These data do not prove that alterations in this cell population are the cause of changes noted in airway and in vascular resistance in infants with lung disease. They do identify, however, a new area of potential investigation in these disease states.

One of the major problems for patients with pulmonary fibrosis is their inability to adequately ventilate their lungs. This results from a loss of elasticity which has generally been thought to be due to increased and defective collagen within the interstitium of the lung. Recent results, however, indicate that contractile force is present within the parenchyma of the normal lung, and this force is greatly increased in pulmonary fibrosis. Muscle tissue within the lung has been excluded as a possible source of this contractility and it appears to be the result of the myofibroblast, a cell that is found in small numbers in the normal lung and in greatly increased numbers in the fibrotic lung. Unlike that produced by collagen, the increased compliance (or "stiffness") which is produced by these cells is reversible, suggesting that treatment might possibly result in relaxation of this cell, reduce its contribution to the ventilatory defect, and thereby provide relief to these patients.

IV. REPORTS ON WORKSHOPS, MEETINGS AND OTHER ACTIVITIES

A. Workshop Reports

Report of Workshop on Cardiopulmonary Resuscitation:
The Role of Lung Mechanics
NIH Publication No. 82-2331

B. Other Reports

National Heart, Lung, and Blood Institute, Division of Lung Diseases Program Report: Fiscal 1981
(for limited distribution)

Report to National, Heart, Lung, and Blood Advisory Council
November 23-24, 1981 (for limited distribution)

V. MAJOR PROBLEM AREAS

A. Endocrine and Metabolic Functions of the Lung

Basic research on the endocrine and metabolic functions of the lung continues to represent an important direction for the pulmonary field. It is clear that the cells and enzyme systems of the pulmonary vascular bed change the biologic activity of a variety of substances presented to them via the pulmonary circulation. Results from this research have already begun to have clinical application;

basic science is certain to continue to provide important new findings in this area.

B. Mechanisms of Acute Lung Injury in Adult Respiratory Failure

Adult respiratory failure affects approximately 150,000 adults each year, a number which is likely to increase with advances in the treatment of trauma victims and in management of severe medical and surgical complications. Survivors become subject to risk of respiratory failure which has a mortality rate of over 50 percent. After years of research on mechanical methods for ventilation, this field has now focused on investigation of more basic aspects of this condition, in particular, the cellular and metabolic aspects of lung injury. Through better understanding of the structural biochemical, and physiologic mechanisms of acute lung injury, better methods for diagnosis and management of patients with respiratory failure may soon be possible.

C. Training

The Division of Lung Diseases continues to draw both young basic scientists and clinical investigators into our training programs. In 1981, over 60 percent of the total number of postdoctoral trainees were young scientists with M.D. degrees. The clinical investigator award continues to provide an important mechanism for support of the M.D. scientist.

D. Prevention of COPD

Chronic Obstructive Pulmonary Diseases is one of the fastest rising causes of mortality in the United States. A program for early detection of the disease may be an effective means of reversing this trend. Although pulmonary function tests that may serve to identify early abormalities are now available, studies must be undertaken in small pilot groups to establish the effectiveness of abnormal pulmonary function measurements as early predictors of clinical disease, and to establish the ability of various interventions to slow the course of the disease process.

ANNUAL REPORT

DIVISION OF BLOOD DISEASES AND RESOURCES
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

October 1, 1981 through September 30, 1982

The Division of Blood Diseases and Resources plans, directs, and evaluates the NHLBI's research grant, contract, and training programs in hematology, hematologic diseases (except malignancies of the blood, and immunologic and other disorders of the white blood cells), and blood resources. These programs include basic research, prevention, applied research and development, clinical trials, education, demonstration, and control activities. The Division monitors current activities and national needs and seeks to develop and support research into the causes, prevention, diagnosis, and treatment of diseases of the blood. Research on the use of blood and blood components and the management of the nation's blood resources are also encouraged.

THROMBOSIS AND HEMOSTASIS

The program in hemostasis and thrombosis supports basic and applied research on all aspects of the coagulation and fibrinolytic systems, including the biochemistry and physiology of clotting factors and inhibitors, platelets, and the vessel wall. Included in this program is the interrelation between these systems and other plasma protease systems, such as the kinin and complement systems. Aspects of hemophilia research, including improvements in methods of carrier detection and genetic counseling, are supported by investigator-initiated grants.

Other research areas include the development of specific blood tests designed to identify patients at high risk for developing venous thrombosis, the development of animal models for thrombosis research, and the relation of von Willebrand's disease and thrombosis to atherogenesis.

RED BLOOD CELL DISORDERS

The program on red blood cell disorders includes research on bone marrow function, on the collection and purification of erythropoietin, on hemoglobin synthesis, structure and function, and on the thalassemias and their treatment, including clinical investigation of chronic iron overload and the use of iron chelation therapy for patients who require repeated transfusions for treatment of thalassemia major.

BLOOD RESOURCES

The Blood Resources Program supports research related to blood banking, improvement of methods of blood fractionation, development of new fractionation products, improvement of the storage of blood and blood products, proper use of blood components in therapy, development of blood substitutes, and elimination of the hazards of blood transfusion, with special emphasis on posttransfusion hepatitis.

SICKLE CELL DISEASE

The Sickle Cell Disease Program supports basic, clinical, and applied research aimed at increasing understanding of the pathophysiology of sickle cell disease. Basic research is directed at globin synthesis, cell membrane function, blood flow characteristics, conformational and structural studies of sickle hemoglobin, and investigation of antisickling agents. Areas of clinical investigation include techniques for antenatal and neonatal diagnosis, management of complications, extracorporeal techniques for drug therapy, and the clinical course, or natural history, of sickle cell disease. The Program also supports continuing education programs for professionals and the public.

RESEARCH TRAINING AND DEVELOPMENT

These programs are directed toward the development of investigators for research in such areas as thrombosis, hemostasis, red blood cell disorders, sickle cell disease, blood resources, and blood-bank-related sciences for the purposes of prevention, detection, and treatment of diseases and disorders of the blood. Research training may be in fundamental and clinical research disciplines related to blood, blood diseases, and blood resources.

HIGHLIGHTS

Blood Resources and Red Blood Cell Disorders

A rapid and simple method for the separation of relatively young red blood cells from ordinary fresh or previously frozen blood units has been developed. Initial clinical studies have shown that the half-life of these young cells in the circulation is 40 to 42 days. The half-life of all red cells (mixed young and old) in a unit of blood is 29 days. Because young red blood cells remain in the circulation longer, patients with Cooley's anemia and other dyserythropoietic anemias can be transfused less frequently and, therefore, may accumulate excess iron less rapidly. A long-term clinical trial is in progress to determine the reduction in annual blood requirements and in the rate of iron accumulation in carefully selected patients.

Blood Resources

Data from studies supported by the Division provide the first comprehensive information about blood collection, processing, and use by blood centers and hospital transfusion services in the United States. These data, for 1979 and 1980, show that approximately 3,000,000 patients received whole blood or red cells in each of those years as a part of their therapy; that one of every 13 hospitalized patients received blood therapy; and that between 5,500,000 and 6,000,000 Americans had whole blood or a blood component drawn either as a donation or as part of their therapy.

Prenatal Diagnosis for Sickle Cell Anemia

Methods for prenatal diagnosis of sickle cell anemia are improving rapidly. Until recently, the diagnostic procedures required sampling fetal blood, at considerable risk to the fetus. New techniques have enabled the use of amniotic fluid, which can be more safely sampled.

The earliest technique involved the use of restriction-endonuclease analysis to detect a polymorphic Hpa I site present in 70 percent of the American Black population. Though safe and useful, this indirect approach required family studies to establish the relationship of the polymorphism to the HbS gene. More recently, the enzyme Dde I was shown to detect the sickle mutation directly and has been a major advance as it is specific and accurate, permits distinction between normal and sickle hemoglobin genes, and permits the use of amniotic fluid rather than fetal blood.

Within the past few months, a new and sensitive assay has been described using the enzyme Mst II. A drawback of the Dde I assay is that the amniotic cells must be cultured to provide a sufficient amount of DNA for analysis. In contrast, this new assay with Mst II is so sensitive that it can be applied to uncultured amniotic fluid cells. This shortens the time needed for the test from five weeks to two weeks and drastically reduces the complexity and cost. The availability of a simple prenatal test increases the potential for widespread application.

Platelet Activation

Platelets perform numerous functions in the process of coagulation. Elucidation of these functions will enable advances in understanding normal and abormal clotting. The activation of platelets is known to involve shape change, the extension and retraction of pseudopodia, spreading and ruffling of the hyalomere, and the secretion of storage granules. It has been hypothesized that platelets contract when activated, and that this contraction causes the outflow of pseudopodia and the release of dense bodies and alpha granules.

Recent findings, however, suggest that platelets do not contract. Using video-enhanced differential interference contrast microscopy at a magnification of 7000X, researchers have observed the process of activation. During platelet spreading, aggregation, and secretory events lasting 25 to 30 minutes, there was no indication of platelet contraction. The microtubule band did not decrease in diameter when contraction was expected to occur. When platelets were treated with calcium ionophore, secretion was accelerated but, again, there was no indication of contraction. Thus, there appears to be fairly strong evidence that platelet contraction of the cytoplasm during activation does not occur and that the apparent concentration of organelles centrally is a result of the outflow of cytosol from around the inclusions.

WORKSHOPS, TASK FORCES, AND MEETINGS

The Division sponsored the following meetings during this fiscal year.

Conference on Psychosocial Aspects of Sickle Cell Disease	January 29, 1982 Bethesda, Maryland
Conference on Coagulation, Cancer, and Inflammation	September 8-10, 1982 Airlie, Virginia
International Symposium on Blood Substitutes	September 29-October 1, 1982 San Francisco, California

NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
DIVISION OF EXTRAMURAL AFFAIRS
Annual Report
October 1, 1981 - September 30, 1982

The Division of Extramural Affairs is responsible for formulating, coordinating and evaluating Institute-wide policies and procedures for research contracts, grants, and training programs. The Division is the focal point for providing advice to the Director, NHLBI, on research contract, grant, and training program policy and procedure. It also represents the Institute on overall NIH extramural grant and collaborative program policy committees, coordinates such policies within NHLBI, and coordinates all of the Institute's research and training program activities with the National Heart, Lung, and Blood Advisory Council. Other major responsibilities of the Division include: (a) grant and contract management and coordinative services for the Institute, (b) initial scientific and technical merit review of certain competing grants and all research contracts for the Institute, (c) committee management functions for the entire Institute, (d) primary role with Advisory Council, and (e) key role in training and orientation of professional extramural staff.

The Division continues to serve as the primary liaison to the National Heart, Lung, and Blood Advisory Council, and has continued to develop procedures and mechanisms to facilitate the Council's review of programs and applications and the review, approval, and implementation of new initiatives and program plans. The Council meets at least four times a year. Several Council working groups have been established and these continue to contribute significantly to the operation of the Institute. Some of these working groups emphasize application review while others emphasize program review and development.

During FY 1982 the Division continued to provide a broad range of services for the entire Institute. These included:

1. Initial scientific and technical merit review of competing research grants, contract and training proposals.

2. Management functions for research grants, contracts, and training/manpower awards.

3. Maintenance of official files for all grant and contract programs, including regular updating to keep them current.

4. Receipt of all final reports and accomplishing the close-out of terminated grants and contracts.

5. Management of functions and activities of the National Heart, Lung, and Blood Advisory Council including coordinating and assisting with the preparation of the Advisory Council's Annual Report.

6. Preparation of official and summary minutes of Council meetings and summary statements of Special Council actions.

7. Preparation of review materials for Council, staff, and Institute initial review groups and proper documentation of their recommendations and actions.

8. Operation of the Program Policy and Procedure Office.

9. Committee management functions.

10. Training and orientation of extramural staff.

11. Representation of the Bureau (Institute) at a variety of central NIH functions, e.g., EPMC, GMAC, RCC, and RPC.

In December 1981, the Institute established a panel of senior scientists with extensive experience in cardiovascular research to investigate an allegation that an investigator at the Brigham and Women's Hospital had falsified research data. This investigator also served an important role in a cooperative study supported by the Institute entitled "Animal Models for Use in the Assessment of Therapy to Protect Ischemic Myocardium" (AMPIM). Dr. Howard E. Morgan served as Chairman of the Panel and Dr. Jerome G. Green, Director, DEA, served as Executive Secretary for the Panel. The Panel conducted an extensive and detailed inquiry into this matter and presented a report of its findings, conclusions, and recommendations to the Acting Director, NHLBI, on June 29, 1982. The Panel's investigation and its report of findings, conclusions, and recommendations represents one of the Institute's most extensive and intensive efforts, to date, in the area of scientific misconduct. The Panel's report is now in the office of the Director, NIH. It is expected that final action will occur in the fall of 1982.

Review Branch

The initial scientific and technical merit review of grant applications and contract proposals has continued to be the main focus of Review Branch activities. In FY 1982, applications were reviewed for:

Pulmonary Academic Awards
Preventive Cardiology Academic Awards
Clinical Investigator Awards
Institutional National Research Service Awards
Minority Hypertension Research Development Summer Program

Supplemental Grants for:

Comprehensive Sickle Cell Center Program
National Research and Demonstration Center Program
Clinical Trial Grants
Research Demonstration and Dissemination Project Grants
Specialized Centers of Research Programs in Hypertension, Ischemic Heart Disease, Arteriosclerosis, and Thrombosis

Solicited Grants(RFAs) for:

NHLBI-81G-D: Demonstration and Education Research in Heart, Blood Vessel, Lung, and Blood Disease and Blood Resources.

NHLBI-DLD 82G-A: Immune Responses in the Lung which Underlie Interstitial Pulmonary Fibrosis.

NHLBI-DLD 82G-B: Specialized Centers of Research in Adult Respiratory Failure.

NHLBI-DLD 82G-C: Structural and Functional Changes Associated with Respiratory Muscular Fatigue.

NHLBI-DHVD 82G-E: Identification of Cells in Atherosclerotic Plaques.

NHLBI-DHVD 82G-F: Analysis of the Roles of Genetic and Developmental Factors in the Etiology and Progression of Essential Hypertension.

NHLBI-DHVD 82G-G: Image Enhancement Techniques for Visualization of Coronary Arteries and Coronary Bypass Grafts.

NHLBI-DHVD 82-G-H: Mechanisms of Calcification of Prosthetic Materials in the Cardiovascular System.

NHLBI-DBDR 82-G-I: Use of Perfluorochemicals in Basic and Applied Research.

Other Unsolicited Grant Programs:

Program Projects
Clinical Trials
Research Demonstration and Dissemination Projects
Conferences
Education Projects

In FY 1982, numerous new and renewal contract proposals were reviewed. In addition, the types of contract initiatives issued by the NHLBI for FY 1982 were:

NHLBI-82-1: Maintenance of Chimpanzee for Hepatitis Research.

NHLBI-82-2: Non-Human Primate Resource (CYNOMOLGUS)

NHLBI-82-3: Development of Implantable Integrated Thermally Powered Ventricular Assist System.

NHLBI-82-4: Development of Diffusion Strategies Among Culturally Diverse Populations 8(a).

NHLBI-82-6: Planning & Implementing the Smoking & Nutrition Education Program.

NHLBI-82-7: Cardiac Arrhythmia Pilot Study - Coordinating Center.

NHLBI-82-8: Cardiac Arrhythmia Pilot Study - Clinical Centers.

NHLBI-82-9: Evaluation of Blood Pressure & Related Data From the NHLBI-HSA Five Sights Demonstration Project 8(a).

In the Division's Review Branch, the Review Processing Section continued to perform its diverse functions in a satisfactory manner with a majority of new personnel. During this fiscal year a total of 555 grant and contract proposals were reviewed by the Branch. Included in this number are 65 Program Project applications.

Grants Operations Branch

The Grants Operations Branch underwent a reorganization within the last two years. The new Branch structure consists of the Office of the Branch Chief, three Grants Management Sections, the Awards Section, and the Council Services and Grants Records Section.

The Branch was involved in the Institute's grant activities from the pre-application (planning) stage to the close-out of terminated grant projects. Branch staff are often contacted by grantee institution staff concerning the best method of presenting consortium budgets, requests for research patient care costs, alterations and renovations, etc. The Branch was also actively involved in site visits, Council preparation, post-Council - pre-award budget negotiations, issuance of award notices (more than 4,000 totaling in excess of $413,000,000 for all the Institute programs) as well as the post-award fiscal and administrative management of those awards. The Branch served as an important interdivisional coordinator, and senior representatives of the Branch continued to be effective in assisting NIH-OD staff in the development and analysis of NIH grant policy and procedure. The Branch's continued efforts to improve management practices and procedures have undoubtedly resulted in considerable dollar savings for the Institute. Recent renovations have contributed to a much more effective working environment in the Awards Section and the Council Services and Grants Records Section.

Selected staff of the Grants Operations Branch were involved in planning and coordinating one day workshops for the Health Scientist Administrators and Grant Managers of the NHLBI. These workshops were held in October 1981 at the Heart House and were designed to increase the awareness of and sensitivity to the roles and responsibilities of the Health Scientist Administrators and Grant Managers. From all accounts, the workshops were a complete success, and it is likely that similar workshops will be carried out in the future.

The Awards Section is responsible for the preparation of all award notices and grant encumbrance lists. The Section also receives, reviews, and files various documents pertaining to NHLBI grants and

awards (e.g., activation notices, and statement of appointment forms). This Section is responsible for monitoring payback required of National Research Service Award trainees and for recording changes (budget period dates, change of P.I., etc.) for all active grants. The Awards Section is now planning to automate a major portion of its activities. Computer terminals with printers will be installed, thus allowing the Institute to issue formal award notices with only a two-day processing delay. The current delay is approximately two weeks.

The Council Services and Grants Records Section continues to improve maintenance of official grant records (pending, active and terminated). The Section files day-to-day correspondence and is responsible for daily update of the file charge-out system. This Section is also responsible for the preparation of Council Books (the May 1982 Council involved nearly 1,400 summary statements) and for the duplication of some summary statements and supplemental material. Further, the Section is responsible for distribution of Council Books and preparation of the Council meetings rooms. The Section also provided information, duplicated from the official grant records, to the Institute's Privacy Act and Freedom of Information Coordinators.

Contracts Operations Branch

The Contracts Operations Branch, responsible for the administrative and business management aspects of the NHLBI contracts program, continues to be involved in a variety of activities: presolicitation, solicitation, evaluation of proposals, determination of the competitive range, negotiation, award, post-award administration, termination and closeout of completed contracts awarded by the Institute.

During FY 1982, fifteen solicitations were prepared and released by the Institute. As a result of these solicitations, it is expected that thirty-six new contract awards will be made. It should be noted that the Institute is trying to increase the participation of small business firms in its contracts program and that four of the fifteen solicitations have been set-aside for small business firms. There has been some decline in the number of active contracts with the completion of the Beta-Blocker Heart Attack Trial and the Multiple Risk Factor Intervention Trial for the Prevention of Coronary Heart Disease. The number of active contracts will be approximately 225, with a value of about $60,000,000.

The Department has continued its interest in the even distribution of contract awards throughout the fiscal year, with particular concern for the avoidance of last minute buying. In conjunction with this, the procurement planning schedule which was developed at the outset of FY 1982 was updated and is submitted to the Department on a quarterly basis. This schedule was maintained to assure an even distribution of awards and to guard against exceeding the maximum goal of 28.6 percent for fourth quarter awards and 12 percent for September awards established by the Department. Our latest estimate suggests that only 19 percent of our awards will be made in the fourth quarter.

The Department's program requiring certification of Contracting Officers and Contracts Specialists remains in effect. Except for one relatively new member of the staff, everyone in the COB required to be certified has either received the appropriate certification or has submitted an application for certification. Certification supposedly reflects a certain level of professionalism; the effective working relationship which exists between program and contract staff within NHLBI indicates that such professionalism does indeed exist.

The contract operations of NHLBI were reviewed by PHS procurement staff during December 1980 and January 1981. The final report of findings were issued on January 8, 1982, and it contained four recommendations. The conclusion reached by the reviewers was that the NHLBI contracts program was managed satisfactorily. A response was prepared by NHLBI which contained a detailed account of actions taken on certain items of the report's recommendations or a considered appraisal of why others could not realistically be adopted. Our response was considered very professional by PHS.

As indicated in last year's report, we centralized most of the responsibilities for closure of expired contracts in the Lung Diseases Contracts Section. This change relieved Contracts Specialists of much of the responsibility in this area, allowing them to devote more time to the negotiation, award, and post-award administration of contracts. This centralization has worked quite well. The Division of Contracts and Grants recently apprised us that in comparing our performance in contract closeout responsibilities with other BID's, that our office has performed outstandingly.

Administrative Activities

During the past year the Division has experienced several key changes of personnel. In March 1982, Dr. Henry Roscoe was appointed Deputy Director, DEA. He occupies a dual position, also serving as Chief, Office of Policy and Procedures.

In January 1982, Mr. John Turlik retired as Chief of the Contracts Operations Branch. Mr. Robert Carlsen was appointed as the new Chief, Contracts Operations Branch on January 24, 1982. Mr. Robert Best was selected to serve in the dual position of Deputy Chief and Section Chief, DHVD Contracts Section in July 1982. Fortunately, the Division has managed to fill quickly most vacancies. Efforts are underway to recruit an Executive Secretary for the National Heart, Lung, and Blood Advisory Council.

The space for DEA is spread over four floors in the Westwood Building, and in addition the majority of the Contracts Operations Branch staff are located in the Federal Building. While most of the staff have adequate space to perform their duties, the Council Services and Grants Records Section, GOB is quite cramped in the 4th floor annex. To alleviate this problem, we have explored ways to better utilize the space available and have ordered new filing equipment for this purpose. The COB staff located

in the Federal Building suffers the inconvenience of separation from their Branch Chief and Division Director and have a continuing problem with malfunctioning heating and air conditioning units.

In July 1982, the Public Health Service conducted an audit of the Institute's Scientific Review and Evaluation Awards. These awards are administered and managed by the Division with an annual budget of approximately $1.4 million. This review also included four other BID's--NCI, DRG, DRR and NINCDS. The PHS expects to issue their final report to the Director, NIH before the end of FY 1982.

INTRAMURAL RESEARCH

THE NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

ANNUAL REPORT

October 1, 1981 - September 30, 1982

INTRAMURAL RESEARCH

Project Reports

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Annual Report
Section on Enzymes
Laboratory of Biochemistry
National Heart, Lung, and Blood Institute
October 1, 1981 - September 30, 1982

A. Regulation of Protein Turnover

(a) Inactivation of Enzyme by Mixed Function Oxidation Reactions

(i) Specificity of the Inactivation Reaction. Previous studies in this laboratory indicated that the degradation of glutamine synthetase (GS) in Escherichia coli is a two-step process involving inactivation followed by proteolysis. Inactivation of GS is dependent upon O_2 and Fe(III) and is catalyzed by any one of the following mixed function oxidation systesm: (1) NADH-diaphorase; (2) NADPH, cytochrome P-450 reductase and P-450 (LM_2) from rabbit liver microsomes; (3) redoxin reductase and redoxin $\pm$ $P\text{-}450_c$ from Pseudomonas putida; (4) a non-enzymic system comprised of ascorbate, and Fe(III). Further studies show that GS inactivation is catalyzed also by xanthine oxidase + hypoxanthine and that inactivation by this system is greatly stimulated by either ferredoxin, P-450, redoxin, FMN, or menadione.

(ii) Generality of the Inactivation Reaction. Of 23 enzymes tested, the following enzymes were readily inactivated by either the NADH-diaphorase system or the rabbit liver microsomal NADPH-cytochrome P-450 system: alcohol dehydrogenase from Leuconostoc mesenteroides or yeast; creatine kinase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase and lactate dehydrogenase from rabbit muscle; GS from E. coli or rat liver; and, phosphoglycerate kinase from yeast. Aspartate kinase III from E. coli was also inactivated by the diaphorase system; its inactivation by the P-450 system has not been tested.

The following enzymes were not inactivated: acetate kinase, aldolase, alkaline phosphatase (E. coli) α-amylase, carboxypeptidase A, fructose-1,6-bisphosphatase, β-galactosidase, glucose-6-phosphate dehydrogenase, β-glucuronidase, hexokinase (yeast) lysozyme, malate dehydrogenase (heart).

The inactivation of pyruvate kinase, GS and phosphoglycerate kinase by both mixed function oxidase systems are dependent upon NADH (or NADPH), O_2, and Fe^{3+}, and are inhibited by Mn^{2+}, catalase, and EDTA. It may be significant that all enzymes inactivated by the mixed function oxidation systems are either kinases or dehydrogenases, i.e., they have substrate binding sites for either ATP, NADH or NADPH. In addition, several of them are known to require a divalent cation for activity and to possess a histidine at the catalytic site.

(iii) Site of the Inactivation Reaction. The inactivation of GS is associated with the loss of only one of sixteen histidine residues in each subunit of the enzyme. The histidine modification leads to the formation of carbonyl-containing derivative as judged by its reactivity with phenylhydrazine and o-phenylenediamine reagents, and, after acid hydrolysis, to the formation of a new acetic amino acid. The possibility that histidine might be a common target for mixed function oxidative modification (inactivation) of enzymes is suggested by the finding that a single histidine residue per subunit is also destroyed during the inactivation of P-glycerate kinase by the ascorbate model system, and also by the demonstration in other laboratories that one histidine is lost during the autoinactivation of erythrocyte superoxide dismutase (E. K. Hodgson and I. Fridovich, 1975, Biochemistry 14, 5294).

To facilitate the isolation and identification of a peptide fragment that

contains the modified histidine, the plasmid from an E. coli strain that overproduces GS (from the laboratory of Dr. Boris Magasanik) was transferred into a new strain which when grown in the presence of radioactive histidine produces GS labeled only in the histidine residues. [^{14}C]Histidine labeled GS was isolated from this strain of E. coli and was inactivated by the ascorbate oxidation system. Following fragmentation of the inactive GS by treatment with cyanogen bromide, a small peptide containing the modified histidine residue was isolated by HPL chromatography. The structure of the peptide, which is presumably at the catalytic site of GS, is under investigation.

(iv) Mechanism. The fact that either ascorbate + Fe(III), or Fe(II) alone, will catalyze inactivation of GS in the presence of O_2, and the fact that all inactivation reactions are inhibited by catalase, suggests that H_2O_2 and Fe^{2+} are intermediates in this reaction. This possibility is supported by the observation that all mixed function oxidation systems tested are able to catalyze the reduction of Fe(III) and also by the fact that GS is rapidly inactivated by a mixture of Fe(II) and H_2O_2 under anaerobic conditions. Unclear, however, are the mechanisms by which Fe(III) is reduced and by which H_2O_2 is formed. The attractive hypothesis that Fe(III) is reduced to Fe(II) by superoxide anion and that Fe(II) reacts with H_2O_2 to generate hydroxyl radical via the well known Haber-Weiss reaction is not supported by the observations that neither superoxide dismutase nor free radical scavengers inhibit inactivation of GS by the ascorbate system or by the putida redoxin NADPH-linked P-450$_c$ mixed function oxidation system.

Superoxide dismutase and free radical scavengers do inhibit inactivation reactions catalyzed by the NADH-redoxin reductase-redoxin system (in absence of P-450) and by the xanthine oxidase-ferredoxin systems. In the xanthine oxidase systems, H_2O_2 rather than O_2^- is probably involved in Fe(III) reduction, because catalase, but not superoxide dismutase, inhibits Fe(III) reduction. The addition of P-450$_c$ to the mixed function oxidase system composed of redoxin reductase + redoxin or of xanthine oxidase + redoxin (or ferredoxin) leads to significant stimulation of the inactivation reaction and also to a decrease in the sensitivity of inactivation to radical scavengers. In contrast, the reduction of Fe(III) by the diaphorase system is insensitive to either superoxide dismutase or catalase. The fact that only one of sixteen histidine residues per subunit is modified, suggests that the inactivation reaction is a highly site-specific process. Therefore, the failure to observe effects of superoxide dismutase or radical scavengers under some conditions might be due to inaccessibility of these reagents to the enzyme site at which the activated oxygen is generated.

(v) Regulation of the Inactivation Reaction. The inactivation of GS by mixed function oxidation systems is affected by both the state of adenylylation of the enzyme and by the substrates, ATP and glutamate. Substrates protect the unadenylylated enzyme from inactivation, but enhance the inactivation of the adenylylated enzyme. Similarly, ATP or phosphoglycerate can protect phosphoglycerate kinase from inactivation by the NADH-diaphorase system, or by the ascorbate system. Protection of enzymes from inactivation by their substrates may therefore be a general phenomenon and might be the basis of an important physiological control mechanism. In the absence of its substrate, an enzyme is nonfunctional and is therefore expendable; so it is destroyed by means of inactivation and subsequent proteolysis. Additional regulation could be achieved through the control of catalase and superoxide dismutase activities.

(vi) Quantitation of the Histidine Modification. Taking advantage of the fact that the oxidative inactivation of GS is associated with the conversion of a histidine residue to a carbonyl derivative, a method was developed for the quantita-

tion of the modified (inactive) form of enzyme in various preparations. The method is based on the fact that the molar absorptivity of the 2,4-dinitrophenylhydrazone derivatives of partially inactivated enzyme preparations (obtained by controlled treatment of GS with the ascorbate oxidation system) is proportional to the extent of inactivation (and to the loss of histidine residue). The specific activity of apparently homogeneous preparations of GS isolated from ten different batches of cells varied from 80 to 120 units/mg. When these preparations were analyzed by the above methods, it was found that the amount of modified (inactive) GS present was inversely proportional to the specific activity. Because Mn^{2+} inhibits completely all mixed function oxidase catalyzed reactions tested, and because Mn^{2+} is present at all steps in the purification of GS, it is unlikely that inactivated enzyme is produced during isolation. Therefore, variations in specific activity of different enzyme preparations reflect differences in the amounts of inactivated enzyme present in the cells at the time of harvest; this supports the view that inactivation of GS is a physiologically significant process.

The spectral perturbation elicited by the binding of each one of the four major dye components in commercial preparations of Cibacron blue to native GS is different from the perturbation obtained with ascorbate inactivated GS. In the presence of dye fraction II, the difference spectrum obtained between native and either 50% inactivated or fully inactivated GS exhibits a simple maximum at 660 nm, the amplitude of which is proportional to the amount of inactive enzyme. This dye fraction can therefore be used to quantitate the amount of inactive enzyme in different enzyme preparations.

(vii) Neutrophil Studies. Studies in other laboratories have shown that mature polymorphonucleic leukocytes (neutrophils) are scavenger cells in higher organisms which are capable of ingesting and killing bacteria. These cells can be activated by a variety of compounds including bacterial chemotatic factor fmet-leu-phe or phorbol myristate acetate (PMA) to undergo a respiratory burst, which is accompanied by the generation of activated oxygen species. This and the fact that bacteria containing high concentrations of Fe(III) are most sensitive to killing by neutrophils, suggested that inactivation of key enzymes by a mixed function oxidase type of reaction may play a role in the bacteriocidal activity of neutrophils. Preliminary studies carried out in collaboration with Drs. H. Weissbach and H. Fliss of the Roche Institute of Molecular Biology, Nutley, New Jersey, were made to test this hypothesis. When GS was incubated with neutrophils, 35-45% of the enzyme was inactivated during the period of oxygen burst; no inactivation occurred in the absence of oxygen burst. Similar results were obtained when whole E. coli cells were incubated with neutrophils. In this case, the intracellular levels of GS were determined, in situ, following lysis of the neutrophils and permeabilization of the E. coli with nonionic detergent. No inactivation was observed in the absence of oxygen burst.

These results suggest that bacterial cell killing by neutrophils may involve inactivation of bacterial enzymes which leads to disruption of cellular metabolism and eventual cell death.

(b) Proteolytic Degradation of Inactivated GS

The post-translational labeling of proteins by chemical modification with radioactive group-specific reagents (viz [^{14}C]formaldehyde) is a common technique for the preparation of labeled substrates used in proteolytic assays. Unfortunately, such modifications can alter the susceptibility of the protein to proteolytic attack. To avoid this kind of artifact in the present study, uniformly ^{14}C-labeled GS was isolated from cell extracts of E. coli that had been grown on a

medium containing a mixture of ^{14}C-labeled amino acids. A portion of the homogeneous GS thus obtained was converted to the inactivated form by inactivation with the ascorbate-O_2-Fe(III) oxidase system. Proteolytic degradation of the native [^{14}C]GS and the inactive [^{14}C]GS preparation was followed by measuring the protease-dependent release of trichloroacetic acid soluble radioactivity. Using this technique, a novel protease that catalyzes the degradation of inactivated GS ten times faster than native GS has been purified 1,500-fold. This partially purified protease(s) is inhibited by aprotinin and PMSF; it is not inhibited by either soybean trypsin inhibitor, α-1 antitrypsin, TLCK, leupeptin, α-2-macroglobulin, or EDTA. These studies confirm the existence in E. coli of a protease that selectively degrades GS after its inactivation by mixed function oxidation systems. This and the above studies support the hypothesis that the turnover of specific enzymes is a two-step process involving inactivation followed by proteolysis.

B. Protein Phosphorylation-Dephosphorylation

(a) Purine Nucleoside-Dependent ^{32}P-Labeling of Brain Protein

Earlier studies (see last year's report) showed that in the presence of [^{32}Pi] or [γ-^{32}P]ATP, extracts of porcine brain catalyze the rapid incorporation of ^{32}P into an endogeneous 35,000 Dalton protein. The labeled protein has now been identified as the glyceryl-3-[^{32}P]phosphate thiol ester derivative of glyceraldehyde-3-phosphate dehydrogenase (G3PDH). It is presumed to be formed either by direct interaction of glycerate-1,3-[1,3-^{32}P]bisphosphate with the sulfhydryl group at the catalytic site of G3PDH or by oxidation of the G3PDH-glyceraldehyde-3-[^{32}P] phosphate complex. Studies with [^{14}C]adenosine indicate that it serves at the source of the glyceraldehyde-3-phosphate and suggests that purine nucleoside metabolism via the pentose-P pathway may play an important role in the energy metabolism of brain.

(b) Phosphorylation of Lens Proteins

Protein kinase activity has been demonstrated in both the cortical and nuclear fractions of bovine lens. Histone II-AS and endogenous proteins can be phosphorylated. In the cortical fraction, a 51,000 Dalton protein is phosphorylated. In the nuclear fraction, proteins of 17,000, 20,000, 22,000, < 10,000 and 61,000 Daltons are phosphorylated. The first three are presumed to be subspecies of crystallin. Purified preparations of α, β, and γ crystallin were not phosphorylated by either the cortical, nuclear or rabbit muscle cAMP-dependent protein kinases. The presence of phosphoserine or phosphothreonine could not be demonstrated in any of the crystallin preparations.

(c) Dephosphorylation of Phosphotyrosyl Groups of Proteins

A search for enzymatic activities in cells which hydrolyze protein phosphotyrosine residues was carried out utilizing a novel phosphotyrosyl protein substrate, phosphotyrosyl GS. A potent protein phosphotyrosyl phosphatase (PTPase) activity in both normal and malignant cells was observed. The PTPase activity in Ehrlich Ascites Tumor cells has been partially purified. This activity appears to be specific for proteins containing phosphotyrosine residues and is distinct from protein phosphatase activities which dephosphorylate phosphoserine and phosphothreonine residues. Contrary to some reports, these studies indicate that alkaline phosphatase activities in cells do not play a role in the regulation of the phosphotyrosine content of proteins. In cells in which alkaline phosphatase was induced more than 100-fold of basal levels, there was not a corresponding increase in the PTPase activity.

Annual Report
Section on Intermediary Metabolism and Bioenergetics
Laboratory of Biochemistry
National Heart, Lung, and Blood Institute
October 1, 1981 - September 30, 1982

During the past year investigators in the Section on Metabolism and Bioenergetics have concentrated on (1) the isolation and characterization of seleno-enzymes and seleno-tRNAs, the mechanism whereby the specific biosynthesis of these selenium-containing macromolecules can be maintained distinct from sulfur pathways and attempts to understand at the chemical and biochemical level the roles of selenium as an essential micronutrient; (2) characterization of 8-hydroxy-5-deaza-flavin dependent enzymes of Methanococcus vannielii, the stereochemistry of the electron transfer reactions catalyzed by these enzymes in formate metabolism, hydrogen evolution and utilization and methane biosynthesis and (3) detailed analyses of seleno-tRNAs from Escherichia coli and Clostridium sticklandii with special reference to identity of the selenium modified base(s) and the possible regulatory roles of these seleno-tRNAs in protein synthesis.

A. Bacterial selenoenzymes

Five of the six known bacterial selenoenzymes have been isolated and studied in this laboratory. These are clostridial glycine reductase, a formate dehydrogenase and a hydrogenase from Methanococcus vannielii, nicotinic acid hydroxylase from Clostridium barkeri and an acetoacetyl-CoA thiolase from Clostridium kluyveri. M. vannielii hydrogenase, the recent addition to this list and the first hydrogenase to be characterized as a selenoprotein, contains 4 gram atoms of selenium per mole. This enzyme (M_r 340,000) consists of three types of subunits with molecular weights of 42,000, 35,000 and 27,000. Selenium is located exclusively in the 42,000 M_r subunits and is present in the chemical form of selenocysteine residues. The hydrogenase also contains iron sulfur centers. This enzyme is sensitive to oxygen but can be reactivated by treatment with a thiol and molecular hydrogen. It utilizes the natural cofactor, 8-hydroxy-5-deazaflavin, as electron acceptor.

The selenium-containing thiolase from C. kluyveri, like many other thiolases, has a molecular weight of about 158,000 and consists of four approximately 40,000 M_r subunits. The subunits are of two types that are barely separable as a doublet by SDS electrophoresis. Selenium, which is incorporated in this enzyme during growth of bacteria in 0.5-1 μM selenite, occurs in the chemical form of selenomethionine. The radioactive amino acid from ^{75}Se-labeled enzyme hydrolysates was indistinguishable from authentic selenomethionine by a variety of ion exchange and thin layer chromatographic procedures. Moreover, in the presence of ATP it was converted to the expected product, [^{75}Se]adenosylselenomethionine, by S-adenosylmethionine synthetase. From amino acid compositional analyses, a value of 1 or possibly 2 selenomethionine residues and 44 methionine residues per mole (158,000 M_r) was calculated. The enzyme also contains 4 equivalents of readily alkylatable cysteine plus 12 additional cysteine residues or a total of 16 cysteine residues per mole. No selenocysteine was detected in the enzyme. The occurrence of selenomethionine in a protein that is very rich in methionine raises the question as to whether Se incorporation in this case is specific or merely a sulfur replacement. Also, the lack of an obvious biochemical advantage of selenomethionine over methionine in a protein such as thiolase is a further reason to question specificity. If ^{75}Se-labeled selenomethionine occurs in the thiolase in a unique position rather than

being randomly distributed among the methionine residues, then this would be the first example of the specific occurrence of this seleno-amino acid in an enzyme. To facilitate isolation of selenomethionine containing proteins and peptides, antibodies have been raised to selenomethionine covalently attached to serum albumen. In this case the selenomethionine served as an effective antigen and the antibodies will be attached to a column and used as an analytical reagent.

Selenium occurs in the chemical form of selenocysteine in four selenoproteins that function as redox type catalysts. Three of these, formate dehydrogenase, glycine reductase selenoprotein A and hydrogenase occur in bacteria and the fourth, glutathione peroxidase, occurs in mammals and birds. There is evidence that the selenol form of selenocysteine serves as one of the redox centers of these enzymes. However, nicotinic acid hydroxylase, another bacterial selenoenzyme that is a redox type catalyst, does not contain selenium in the form of selenocysteine residues. Selenium is readily dissociated from the enzyme by heating or by chaotropic agents and, if the enzyme was first inactivated by alkylation, the only product detected is a dialkyl selenide. From this it is concluded that selenium may occur in nicotinic acid hydroxylase as a labile substituent of a ring structure. Another possibility, based on the recent discovery that nicotinic acid hydroxylase also contains molybdenum in the form of a molybdo-pterin cofactor, is that selenium is present as a constituent of this cofactor (instead of a sulfur that occurs in xanthine oxidase molybdo-pterin cofactor). Some support for this idea is the observation that molybdenum, selenium and pterin are dissociated from the enzyme simultaneously and cofractionate when subjected to molecular sieve chromatography.

B. Seleno-tRNAs

Amino acid transfer nucleic acids (tRNAs) modified in the polynucleotide portions of the molecules with selenium have been detected in several bacterial tRNA populations. Preliminary results indicate that seleno-tRNAs also may be natural components of tRNAs from mouse leukemia cells (L1210), mouse liver, kidney and spleen and Ehrlich ascites tumor cells. The ^{75}Se-labeled tRNA preparation from the L1210 mouse leukemia cell line was separated into two hydrophobic ^{75}Se-tRNA species by reversed phase chromatography.

Two anaerobic bacteria, Clostridium sticklandii and Methanococcus vannielii, are especially rich sources of seleno-tRNAs and have been used for detailed study. Five or six separable species of tRNAs in these bacteria contain Se-modified nucleotides and these represent about 5 to 8% of the total tRNA population. Because of the especially high affinity of selenium compounds over sulfur for mercury, the seleno-tRNAs can be selectively enriched by affinity chromatography on Hg-agarose columns. In enriched samples of seleno-tRNAs from C. sticklandii, more than 20% of the total population contained selenium. Concomitant enrichment of glutamate, glutamine, lysine and tryptophan isoacceptor tRNA species indicated these might be seleno-tRNAs. A major seleno-tRNA component proved to be a glutamate accepting species and this was isolated as its amino acylated derivative by reversed phase chromatography. The pure $tRNA^{Glu}$ contained one atom of selenium per active tRNA molecule. Loss of part of the selenium during deacylation treatment resulted in parallel loss of glutamate acceptance activity. The resulting tRNA population could be shown to be a mixture of inactive, selenium-free molecules and fully active seleno-$tRNA^{Glu}$ species. From this it is concluded that the selenium modified base in this seleno $tRNA^{Glu}$ is essential for interaction with its cognate tRNA synthetase. In ribosome binding assays, the seleno-$tRNA^{Glu}$ recognizes both glutamate codons (GAG and GAA) but not incorrect codons.

In Escherichia coli about 2% of the total tRNA population contains Se-modified nucleotides which also are distributed among 5 or 6 separable tRNA species. Earlier studies showed that biosynthesis of these seleno-tRNAs is specific and occurs by a mechanism distinct from the biosynthesis of sulfur modified tRNAs. Separation of these seleno tRNAs under conditions that preserved in vivo aminoacylation followed by rechromatography after deacylation resulted in markedly different elution patterns of the ^{75}Se-labeled tRNAs. Restoration of the original pattern upon enzymic aminoacylation in vitro showed that the seleno tRNAs were active as amino acid acceptors both in vivo and in vitro. In vitro aminoacylation with lysine alone, rather than a mixture of amino acids, shifted the elution position of a major selenium-containing tRNA species (40% of the total ^{75}Se). Similar experiments with glutamate shifted another selenium-tRNA peak that accounted for 10% of the total ^{75}Se. Thus about 50% of the seleno tRNAs of E. coli consist of lysine and glutamate-accepting species. Quantitation with [^{14}C]lysine and [^{14}C]glutamate indicated that about 5% of the total lysine accepting tRNA species and 1% of the total glutamate accepting species are modified with selenium.

High performance liquid chromatographic analysis of the selenium modified moiety in enzymic digests of ^{75}Se-labeled bulk tRNA indicated the presence of a single radioactively labeled peak. This material was isolated in apparently pure form from preparations digested to the nucleoside level and tentatively identified as 5-methylaminomethyl-2-selenouridine. A synthetic procedure for preparation of this previously uncharacterized selenonucleoside was developed. One of the starting materials for this series of reactions is selenourea, a compound that is highly unstable in the light. Although the final product has not yet been obtained in good yield, it exhibited the expected UV absorbance spectrum and chromatographic properties. Coelution of the ^{75}Se-labeled nucleoside isolated from ^{75}Se-tRNAs and the synthetic nucleoside from the HPLC column indicate possible identity. These findings are of particular interest in view of the known occurrence of 5-methylaminomethyl-2-thiouridine as a minor nucleoside in E. coli tRNA^{Lys} and tRNA^{Glu} populations. A single ^{75}Se-labeled nucleoside with the same HPLC chromatographic properties and UV spectrum was detected in enzymic digests of the purified seleno-tRNA^{Glu} from C. sticklandii. This also corresponded to the single component detected in digests of bulk [^{75}Se]tRNA from C. sticklandii. From these results it is tentatively concluded that the selenium modified base, 5-methylaminomethyl-2-selenouracil, is the major or perhaps the only selenium-modified minor base present in E. coli and C. sticklandii tRNAs. Additional seleno-nucleosides were detected in digests of M. vannielii bulk [^{75}Se]tRNA but the major component appears to be the same as that in E. coli and C. sticklandii. Although the tRNAs of all of these bacteria are rich in 4-thiouracil none of the corresponding seleno-base, 4-selenouracil, has been detected.

The location of the 5-methylaminomethyl-2-selenouridyl residue in the seleno-tRNAs (is it adjacent to the anti codon?), effects of this minor nucleoside on various steps of protein synthesis and the enzymology of the selenium modification process are aspects of this general problem that are currently under investigation.

Annual Report
Section on Protein Chemistry
Laboratory of Biochemistry
National Heart, Lung, and Blood Institute
October 1, 1981 to September 30, 1982

Research in the Section on Protein Chemistry consists of studies on the physical and chemical properties of macromolecules of biological interest and on the roles of ligand binding and of protein-protein and inter- and intra-subunit interactions in enzyme catalysis and regulation. The energetics of ligand binding to proteins involves contributions from both ligand-protein and protein-protein interactions. Ligand-promoted changes in protein-protein interactions underlie the phenomenon of cooperativity in ligand binding to proteins and, in addition, give rise to the numerous examples of stabilization and destabilization of protein structures by ligands, metal ions, and other inorganic ions.

Glutamine synthetase, a strictly regulated enzyme in Escherichia coli, is a dodecamer with each subunit containing a catalytic site with two essential divalent cation sites (n_1 and n_2) and a tyrosyl residue which is the site of covalent modification by enzymatically-catalyzed adenylylation-deadenylylation reactions. Studies of the interactions of divalent cations, substrates, substrate analogs, and inhibitors with glutamine synthetase from E. coli have continued. Kinetic studies of Mn^{2+} release from high-affinity n_1 sites have shown that 1 H^+/subunit binds at the fast rate of Mn^{2+} dissociation (presumably to a group within the Mn^{2+} binding cluster) and another H^+/subunit binds after Mn^{2+} release as the protein undergoes a slow conformational transition. Kinetic and equilibrium data indicate that Mn^{2+} binds to n_1 sites of the enzyme in two states (a low- and a high-affinity conformation); active-site ligands stabilize additional conformations of the enzyme which have decreased rates of Mn^{2+} dissociation. Calorimetric studies of the interactions of the substrate analog L-methionine-S-sulfoximine with unadenylylated and adenylylated enzymes have shown that binding enthalpies and proton effects differ markedly, indicating conformational differences between these enzyme forms. Also, L-methionine-S-sulfoximine produces different spectral perturbations on binding to unadenylylated and adenylylated enzymes. The UV spectral perturbations derive from a conformational change that is solely a marker for the occupancy of the single subunit site by active-site ligand. More subtle conformational changes involving pK perturbations of ionizable protein groups without major structural alterations have been detected by calorimetric measurements of glutamine synthetase interactions with substrates and substrate analogs.

Cooperative protein interactions in glutamine synthetase are revealed by the extremely tight but reversible binding of 2 Mn^{2+}, L-methionine-S-sulfoximine phosphate, and ADP formed on each enzyme subunit at pH 7 by phosphorylation of the substrate analog with ATP ($K'_A > 10^{12}$ M^{-1} for ADP). Disruption of the enzyme complex depends on the protonation of 3-4 carboxylate groups per subunit and on structural perturbations produced by increasing temperature and KCl concentration. At neutral pH, the tight synergistic binding of ligands to the active-site of each subunit of glutamine synthetase strengthens both intra- and inter-subunit bonding domains in the dodecamer. This linkage between active-site ligand binding and inter-subunit bonding strength in glutamine synthetase has been found to be quite remarkable. Advantage is being taken of these newly discovered properties to obtain information on the topography of different sites within the glutamine synthetase molecule. Studies with E. coli enzyme are being extended to obtain comparable data for the octameric glutamine synthetase from bovine brain.

Studies of the Zn^{2+} binding domains in aspartate transcarbamoylase from E. coli have been initiated. The stability characteristics of Zn^{2+} binding domains as well as the kinetics of Zn^{2+} release caused by reaction of sulfhydryl groups with mercurial reagents will give valuable information on contact regions between catalytic and regulatory chains.

Annual Report
Section on Metabolic Regulation
Laboratory of Biochemistry
National Heart, Lung, and Blood Institute
October 1, 1981 to September 30, 1982

The research activities of the investigators in the Section on Metabolic Regulation are mainly concerned with the physical and chemical approaches to resolve the mechanisms of enzyme action and its regulation, and to study the antibody-antigen interaction. Currently, the research is concentrated on (1) the regulation of enzymatic activity by a cyclic cascade system and by a Ca(II)·calmodulin complex; (2) preparation of monoclonal antibodies against the adenylylated and unadenylylated glutamine synthetase; (3) mechanistic studies of enzyme action; and (4) development of analytical methods to facilitate biochemical studies. Together, these research programs will provide a better understanding on how biochemical processes work in cells.

I. Regulation of Enzymatic Activity

A. Phosphorylation-Dephosphorylation Cascade

The regulatory properties predicted by the simplified monocyclic cascade model have been verified experimentally using a system consisting of two converter enzymes, a cyclic AMP-dependent protein kinase and a phosphoprotein phosphatase, and a synthetic substrate peptide, Leu-Arg-Arg-Ala-Ser-Val-Ala-Gln-Leu. In addition, the simplified model has been extended to consider the contribution of the concentration of the converter enzyme-interconverter substrate complex. This extended model, as shown both theoretically and experimentally, reveals that the monocyclic cascade is potentially more sensitive to the change in concentration of allosteric effectors than those predicted by the simplified model. Furthermore, the study of the converter enzymes used shows that: (1) The bovine cardiac phosphoprotein phosphatase is a predominantly type-2 phosphatase since it is inhibited by the heat stable inhibitor-2. The results of the inhibition study using ATP and NaF as inhibitors, Mg(II) as modulator, in the presence or absence of Mn(II), indicate that ATP, F^-, Mg(II) and Mn(II) each act by binding to separate sites on the enzymes. (2) The data from the activation of bovine heart cyclic-AMP-dependent protein kinase by cyclic-AMP suggest that only two equivalents of cyclic-AMP are required for the activation of each holoenzyme.

B. Glutamine Synthetase Cascade

1. Construction of a Multicopy Plasmid Vector to Cause Overproduction of UT-UR Enzyme. Glutamine synthetase is regulated mainly by a bicyclic cascade, in which uridylyltransferase (UT) and uridylyl-removing enzyme (UR) serve as the converter enzyme for uridylylating and deuridylylating the regulatory protein, P_{II}, respectively. The covalently modified (P_{IID}) and unmodified P_{II} (P_{IIA}) in turn activates the deadenylylation and adenylylation of the adenylyltransferase, respectively. Purification of the UT-UR enzyme, a bifunctional polypeptide, has shown to be a difficult task due to its low quantity and instability. Therefore, research has been carried out to construct a multicopy plasmid vector carrying the structural gene for UT-UR enzyme (glnD gene). A strain, constructed by cloning the EcoRl fragment of the plasmid JA200/PLC 38-39, overproduces UT-UR by 25 to 30-fold over the wild type strain. However, this level of overproduction is not sufficient to facilitate the purification study. Thus, an attempt to fuse the glnD gene and a highly efficient E. coli promoter, such as the promoter of the lac

operon, is currently in progress.

2. Studies of P_{II} Regulatory Protein. A large quantity of P_{II} protein was purified from an overproducing strain constructed by cloning PVUl generated fragment of the recombinant plasmid PGSl. The purification procedure used is a simplified method which includes a 26% β-mercaptoethanol precipitation step. The purified protein was used for the following studies: (i) X-ray crystallographic data show that the unmodified P_{II} (P_{IIA}) was crystallized in the cubic space group I23. The unit cell contains six molecules. The molecule has a symmetry 222, indicating that the four subunits in each molecule assume tetrahedral structure; (ii) UV spectrum of P_{IIA}, P_{IID} and their difference spectrum were obtained. The difference spectrum is the same as that of UMP. The second derivative spectrum of denatured (in 6 M guanidine) P_{IIA} indicates that the ratio of phenylalanine to tyrosine to tryptophan is 4.8:2.0:0.06. The result is in good agreement with the result of amino acid analysis which shows that P_{II} subunit contains five phenylalanines, two tyrosines, and no tryptophan. Based on the spectral study and the assumption that the extinction coefficient of the UMP moiety is not changed when covalently attached to P_{II}, an empirical formula was derived for calculating the concentration of P_{IIA}, P_{IID} and total P_{II} from the spectrum of pure P_{II} protein. (iii) The amino acid sequence of a tryptic undecapeptide containing the covalently bound UMP was shown to be Gly-Ala-Glu-Tyr(UMP)-Met-Val-Asp-Phe-Leu-Pro-Lys. The peptide, which contains 91% of the total radioactivity of the heat denatured $P_{II}[^{3}H, {}^{31}P\text{-UMP}]_4$, was produced by trypsin digestion of the P_{II} protein and purified by the HPLC method. (iv) Phosphotyrosyl P_{II} was produced by incubating $[^{3}H, {}^{32}P]$uridylylated P_{II} with micrococcal nuclease. Removal of uridine moiety caused P_{IID} to lose its ability to activate the deadenylylation reaction catalyzed by adenylyltransferase, while the phosphotyrosyl P_{II} exhibits only 2% of the P_{IIA} activity.

C. Regulation by Calmodulin

Calmodulin is known to activate a bovine brain calmodulin-dependent cyclic nucleotide phosphodiesterase. The activation mechanism involves the formation of five enzyme-calmodulin complexes which derive from the interactions between cyclic nucleotide phosphodiesterase and various Ca(II)-liganded calmodulin species. Of all these complexes, the predominant species is the enzyme-calmodulin complex containing four Ca(II). The dissociation constant for the calmodulin·$Ca(II)_4$ from the enzyme-Ca(II)-calmodulin complex was previously determined to be 10^{-10} M at pH 7.0, 25°C. This value has been redetermined by measuring the calmodulin activated phosphodiesterase activity, at a given calmodulin concentration, as a function of free Ca(II) concentration. The new value is 1.2×10^{-11} M, indicating that the affinity of phosphodiesterase for the fully liganded calmodulin is almost one order of magnitude higher than previously determined. The error in the previous value was derived mainly from the uncertainty of the concentration of active phosphodiesterase.

II. Mechanism of Enzyme Action

A. Glutamine Synthetase from E. coli

1. Zn(II) Activation and Inactivation of E. coli Glutamine Synthetase. Zn(II) has been shown to support the γ-glutamyltransferase reaction catalyzed by glutamine synthetase at low concentration, but inactivates this enzymatic activity at high concentration range. The results of the kinetic analysis of these two Zn(II) effects suggest that: (i) When Zn(II) binds to the catalytic site of each subunit of the dodecameric enzyme, it supports the activity of that subunit. The dissociation constant for this Zn(II) binding site is 0.22 μM and this binding

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affinity is varied as a function of the fractional saturation of the second Gln binding on each subunit. The value of K_d for the second Gln is 105 mM. When the second Gln site is saturated, K_d for Zn(II) increases to 3.9 μM. (ii) In each subunit there exists a Zn(II) inhibitory site. When six of the twelve subunits are filled with Zn(II) at the inhibitory site, the dodecamer would dimerize or polymerize with other active dodecamers to form a relatively inactive dimer or polymer. The dissociation constant for Zn(II) binding at the inhibitory site is 1.07 μM, and this value is also varied as a function of the fractional saturation of the second Gln site. At saturating levels of Gln (second site), the K_d value for the inhibitory site increases to 39 μM. This high degree of apparent cooperativity is needed to explain the sharp drop in activity as [Zn(II)] increases. However, the alternative explanation, such as Zn(II) induced enzyme precipitation, inactivation caused by substoichiometric Zn(II) binding in the dodecamer, or inactivation due to multiple Zn(II) binding at the inhibitory sites in each subunit, cannot be ruled out. Nevertheless, it should be pointed out that attempts to detect enzyme precipitation has not been fruitful.

2. Identification of Substrate Induced Protein Conformational Changes in Glutamine Synthetase. Based on protein fluorescence changes observed upon substrate binding, it is assumed that these fluorescence changes are derived from protein conformational changes. In an attempt to identify the conformational changes described above, we have adenylylated the glutamine synthetase with a spin-labeled ATP analog, 2,2,6,6-tetramethylpiperidine-1-oxyl adenosine triphosphate (Tempo-ATP). The Tempo-adenylylated glutamine synthetase shows essentially identical catalytic properties, pH profile, and inhibitor susceptibility to those of the naturally adenylylated enzyme. The ESR spectrum of the covalently bound Tempo-AMP reveals that rotational correlation time of the nitroxyl moiety is 7.2×10^{-11} sec which is similar to 8.6×10^{-11} sec determined for the free Tempo-ATP. This observation indicates that the adenylylation site is located on the surface of the protein. Using the paramagnetic effect of Mn(II) on the ESR signal of the spin-labeled Tempo-glutamine synthetase, the distances from the nitroxyl moiety of the covalently bound Tempo-AMP to the structural divalent metal ion binding site (n_1 site) and the catalytic site (n_2 site) were determined when Mn(II) was used as divalent metal ion. In the absence of substrate, the distances from Mn(II) at the n_1 and n_2 sites to the nitroxyl moiety are 19 and 16 Å, respectively. These findings are in good agreement with the data obtained with ^{13}C NMR and fluorescence energy transfer methods. Binding of substrate such as L-Glu causes a reduction of ~ 2 Å for the n_1 to Tempo-AMP distance and a lengthening of ~ 2 Å for the n_2 to Tempo-AMP distance. Addition of ATP to the Tempo-GS·L-Glu complex increases the distances between n_1 and Tempo-AMP, and n_2 and Tempo-AMP by 4 and 3 Å, respectively.

B. Mechanistic Study of Rabbit Skeletal Muscle Actomyosin ATPase

In contrast to the Lynn-Taylor model for actomyosin ATPase which suggests that ATP hydrolysis required the dissociation of ATP·actomyosin complex to ATP·myosin and actin, the results from studies carried out in collaboration with E. Eisenberg indicate that myosin does not have to detach from actin during ATP hydrolysis. In the present work, myosin subfragment-1 (A1) (S-1(A1)) was used to investigate the nature of the rate-limiting steps in the catalytic cycle of acto-S-1. The results obtained at 15°C, in very low ionic strength, suggest that a "six-state" kinetic model is necessary to explain the actomyosin ATPase. In this model, the rate-limiting step occurs after the cleavage of ATP, but precedes the rapid release of Pi. This rate-limiting step may play an important role in determining the velocity of muscle contraction.

C. The Mechanism of Activation of Proteinase B from Yeast

The slow activation of proteinase B in yeast at pH 5 was thought to be due to the degradation of proteinase B inhibitor, I_B, by proteinase A. However, it was shown that when proteinase A was completely inhibited by pepstatin, the activation of proteinase B was not hampered. The present study reveals that I_B can exist in four forms separable by isoelectrofocusing method. The four I_B forms exhibit different susceptibilities to loss of inhibitory effect by pH 5 treatment. The loss of inhibitory effect appears to be the result of a slow, irreversible conformational rearrangement.

III. Immunochemical Studies

Preparation of Monoclonal Antibodies Specific to Various Antigenic Determinants of E. coli Glutamine Synthetase

Monoclonal antibodies specific to adenylyl moiety or specific to a fluorescent analog of AMP, aza-ε-AMP, were obtained by immunizing BalbC mice with AMP-BSA or aza-ε-AMP-BSA conjugate. The antigen-primed spleen cells were hybridized with SP2 myeloma cells and screened for cells producing immunoglobulin which bind the adenylylated glutamine synthetase or aza-ε-ATP adenylylated enzyme. Four clones producing different subclasses (IgM, IgG1, $IgG2_a$, and IgG3) were selected for AMP specific immunoglobulin and two clones producing IgG1 and $IgG2_b$ for aza-ε-AMP specific immunoglobulin. In addition, antibodies recognizing antigenic determinants other than adenylyl moiety were also obtained from mice immunized with E. coli glutamine synthetase. Preliminary work on unpurified antibodies indicates that two monoclonal antibodies, IgM and IgG1, can distinguish between the relaxed form (likely the dissociated subunits) and the taut form of glutamine synthetase, presumably by binding to the subunit interaction site; and one clone produced antibody capable of inhibiting the γ-glutamyltransferase activity while six other did not. For large quantities of antibody production, cloned cells were injected into the peritoneal cavity of mice, ascites fluids were collected and subsequently immunoglobulins were purified by fractional salting-out, followed by DEAE-cellulose chromatography and gel filtration. Further purification and quantitative characterization studies are currently in progress.

IV. Development of Analytical Methods

A. A Continuous Fluorometric Assay for the Deadenylylation Reaction of Glutamine Synthetase

In this method, glutamine synthetase was adenylylated with a fluorescence analog of ATP, 2-aza-1,N^6-ethano-ATP. The deadenylylation reaction, catalyzed by the P_{IID} activated adenylyltransferase, can be monitored by the decrease (~ 40%) in fluorescence intensity at the excitation wavelength of 300 nm with the emission wavelength set at 470 nm. The fluorescence decrease due to the deadenylylation was linear with respect to time up to ~ 70% completion when a saturated concentration of α-ketoglutarate was used, and the initial rate was directly proportional to the concentration of either adenylytransferase or P_{IID} protein.

B. A Further Extension of the Theory of Continuous Variation Method for Determining K_d and Stoichiometry

The continuous variation method for determining the binding constant and stoichiometry has previously been extended to stepwise binding for identical and noninteracting sites using the limiting slope method of Asmus. In the present

work, this method has been further extended to proteins which exhibit cooperative binding effects. The theory is applicable to both the sequential model of Adair and the concerted model of Monod, provided that the total protein and ligand concentration used is much greater than all the ligand dissociation constants.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00201-11 LB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Metabolism of the Branched-Chain Amino Acids

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.: J. M. Poston Research Chemist LB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

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SUMMARY OF WORK (200 words or less - underline keywords)

A study of the metabolism of the branched-chain amino acids has revealed a pathway of metabolism of leucine that is catabolic in bacteria and appears to be synthetic in humans. The pathway depends upon the activity of the enzyme leucine 2,3-aminomutase, which requires adenosylcobalamin as a cofactor. Another enzyme which functions in the pathway is β-leucine transaminase, and yet another appears to be thiolase. The relationship between enzyme activity and various disease states such as pernicious anemia and maple syrup urine disease will be examined.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: The catabolism of the branched-chain amino acids, leucine, isoleucine, and valine, remains incompletely understood. Although much information that is available has been derived from the study of inborn errors of amino acid metabolism, studies of bacterial fermentation and of animal tissues have given additional information about the catabolism of these amino acids. The objectives of this project are to establish the fermentation pathways of leucine and the other branched-chain amino acids, to examine the enzymes responsible for the various metabolic steps in these fermentations, to explore the distribution of these pathways in other species, to examine the enzymes in these other species, and to examine the implications of these pathways in human metabolism.

Major Findings

As previously reported, when cells or extracts of several clostrida, expecially Clostridium sporogenes and Clostridium lentoputrescens are incubated with L-leucine, several metabolic products are formed that are consistent with the metabolic pathway reported in mammals. However, the production of isobutyrate could not be explained by previously postulated pathways. Evidence was accumulated to support the pathway:

$$\alpha\text{-leucine} \rightleftarrows \beta\text{-leucine} \rightleftarrows \beta\text{-ketoisocaproate} \rightleftarrows \text{acetate} + \text{isobutyrate}$$

The first step is catalyzed by the enzyme, leucine 2,3-aminomutase, which requires coenzyme B_{12} [adensylcob(III)alamin]. This enzyme has been partially purified from a variety of sources (clostridia, spinach, potatoes, and sheep liver), but it has proven to be remarkably unstable after partial purification. It has been measured in human liver, hair roots, and leukocytes.

β-Leucine transaminase, the enzyme which converts β-leucine to β-ketoisocaproate, has been examined in sheep liver. The coupled enzyme assay utilized β-hydroxybutyrate dehydrogenase which reduced the keto product of the transamination at the expense of NADH. An activity that promoted the oxidation of NADH and that was dependent upon β-leucine for activity was purified. However, during purification, it was noted that there was decreased dependence upon the coupling dehydrogenase. It became clear that this activity was due to glutamate dehydrogenase and that the apparent dependence of the reaction upon β-leucine was due to its ability to serve as a potent effector of the enzyme.

Glutamate dehydrogenase purifed from sheep liver has been found to be similar to that reported from beef liver in that ADP and leucine are positive effectors and GTP is a potent negative effector. In these studies, β-leucine was shown to be a strong positive effector and this action is due to the L-isomer; the D-isomer is very nearly without effect. Either leucine or β-leucine is synergistic with ADP in causing NADH oxidation. GTP inhibits the oxidation and the inhibition can be overcome with β-leucine. A preparation of commercial bovine liver glutamate dehydrogenase was examined and found to have a similar response to β-leucine.

Sheep liver glutamate dehydrogenase is a very stable enzyme which purifies as a large, relatively amorphous material. When purified in the presence of Triton X-100, it has a molecular weight (by gel filtration) of 210,000. The livers have been stored frozen so that accurate estimation of the distribution in subcellular organelles is not possible, however, it is probable that this enzyme

is mitochondrial. A β-leucine stimulable glutamate dehydrogenase is found in rat liver mitochondria and the well studied bovine enzyme is known to be a mitochondrial constituent.

Because of the contamination of the preparations with this β-leucine-responsive glutamate dehydrogenase, data previously reported for β-leucine transaminase must be disregarded. It appears that the transaminase is less stable than the dehydrogenase, that it is separable from the dehydrogenase, and that it may have a subcellular distribution different from the dehydrogenase. Leucine 2,3-aminomutase is primarily a cytosolic enzyme and it appears that β-leucine transaminase is also cytosolic. This is a more understandable relationship than if the transaminase were mitochondrial for the two enzymes catalyze adjacent steps in the metabolism of leucine.

The product of the transaminase, β-ketoisocaproate, is subject to cleavage to form acetate and isobutyrate. This is presumably a thiolytic cleavage. It has not yet been possible to demonstrate the production of isobutyryl-S-CoA from the β-keto acid. It has, however, been possible to demonstrate that sheep liver extracts and cystosolic preparations of rat liver possess a thiolase which will cleave the coenzyme A thioester of β-ketoisocaproate. This activity is probably not due to the β-ketothiolase that acts on acetoacetic acid since the relative activities toward the two substrates vary quite widely from preparation to preparation. The thiolase activities have been assayed using the loss of absorbance of the magnesium-β-enol maximum at 303 nm. It has not yet been demonstrated that the products of the reaction are isobutyryl-S-CoA and acetyl-S-CoA, but these are the most likely ones.

Proposed Course of Research

The enzymes of the β-leucine pathway will be purified and characterized. To this end, the conditions which yield maximum activity in cell cultures will be established, and sources and tissues which may yield stable enzymes will be examined. The nature of the B_{12} involvement in leucine 2,3-aminomutase will be established and the nature of the other cofactor involvement will be examined. The relation of the thiolase activity for β-ketoisocaproyl-S-CoA to the β-leucine pathway will be examined. The relation of the pathway to the metabolism of other amino acids and to lipids will be examined. The cofactor requirements for the various enzymes will be established.

Relevance to Biomedical Research

This study impinges on several areas of medical concern: the mode of action of vitamin B_{12} in its metabolic roles, the metabolism of amino acids and nutrition. The mode of action of B_{12} is imperfectly understood, but its importance in hematopoiesis and in the maintenance of proper neurological function is exemplified in the disease of its metabolic deficiency, pernicious anemia. Prior to these studies, only two B_{12}-dependent enzymes had been demonstrated in man; leucine 2,3-aminomutase is now the third. Several inborn errors of metabolism are involved with amino acid metabolism and the effects of these errors may be devastating to the well being of humans, especially in the instances of maple syrup urine disease, isovalericacidemia, and disorders of the catabolism of short-chain acids. The β-leucine pathway may be involved in some of the syndromes associated with these inborn errors.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00202-11 LB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Kinetics, Regulation and Mechanism of Biochemical Reactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	0.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

(1) The results of the analysis of Zn(II) activation and inactivation of E. coli glutamine synthetase suggest that in addition to binding at the structural site, Zn(II) binds tightly to the catalytic site and supports the activity of the enzyme. The enzyme also possesses a Zn(II) inhibitory site in each subunit and the Zn(II) inhibition process is highly cooperative. The affinity of Zn(II) binding to the catalytic site and inhibitory site varies as a function of the fractional saturation of the second Gln binding on each subunit. (2) An ESR study of a spin-labeled Tempo-ATP adenylylated glutamine synthetase revealed that the covalently bound Tempo-AMP exhibits a similar rotational correlation time as that of free Tempo-ATP, which indicates that the adenylylation site is located on the surface of the protein. Using the paramagnetic effect of Mn(II) on the ESR signal of the spin-labeled Tempo-enzyme, we showed that the distances between the adenylyl site and the two metal ion sites are changed due to substrate binding and formation of a reaction intermediate. (3) Study of actomyosin ATPase mechanism revealed that a "six-state" kinetic model is required to explain the actomyosin ATPase reaction.

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Project Description

Objectives: (1) Utilizing the physical-chemical approach to study the kinetics, mechanism, and regulation of biochemical activity. In particular, the biochemical action between the substrates, metabolites, and enzymatic activity of glutamine synthetase from Escherichia coli will be elucidated. In addition, fast kinetic techniques which are useful in these studies will be improved. (2) Theoretical analysis of the cyclic cascade system with respect to its properties and function in the metabolic regulation of key enzymes will be continued. (3) Isolation of the regulatory proteins to allow detailed mechanistic studies, and experimentally verify the validity of the cyclic cascade model described in (2). (4) To study the regulatory mechanism of calmodulin activated cyclic nucleotide phosphodiesterase.

Major Findings

I. Analysis of Zn(II) Activation and Inactivation of E. coli Unadenylylated Glutamine Synthetase.

The catalytic activity of glutamine synthetase from E. coli is known to be modulated by divalent metal ions, in addition to that regulated by feedback inhibitors and by covalent modification of the enzyme (Stadtman and Ginsburg, The Enzymes, Vol. 10, 3rd ed., p. 755, 1974). Among the divalent metal ions studied, Zn(II) has been shown to support the γ-glutamyltransferase reaction at low concentration but inactivates this enzymatic activity at high concentration range (E. R. Stadtman and P. Z. Smyrniotis). When the observed reactivity was plotted as a function of total Zn(II) concentration, it yielded a family of Gln concentration dependent "bell shape" curves. In order to interpret this observation, free Zn(II) concentration was calculated for each activity measured using the experimentally determined dissociation constants for Zn·ADP, Zn·Gln, Zn·$(H_2AsO_4)_2$, Zn·$(HAsO_4)$ and the computed solubility product for $Zn_3(AsO_4)_2$. When the data were plotted as a function of free Zn(II) concentration, the activity first increased as a normal binding isotherm followed by a sharp decrease in activity. The analysis of these data suggest that: (i) When Zn(II) binds to the catalytic site of each subunit of the dodecameric enzyme, it supports the activity of that subunit. The dissociation constant for this Zn(II) binding site is 0.22 μM and this binding affinity is varied as a function of the fractional saturation of the second Gln binding on each subunit. The value of K_d for the second Gln is 105 mM. When the second Gln site is saturated, K_d for Zn(II) increases to 3.9 μM. (ii) In each subunit, there exists a Zn(II) inhibitory site. When six subunits are filled with Zn(II) at the inhibitory site, the dodecamer would dimerize or polymerize with other active dodecamers to form a relatively inactive dimer or polymer. The dissociation constant for Zn(II) binding at the inhibitory site is 1.07 μM, and this value is also varied as a function of the fractional saturation of the second Gln site. At saturating levels of Gln (second site), the K_d value for the inhibitory site increases to 39 μM. This high degree of apparent cooperativity is needed to explain the sharp drop in activity. However, the alternative explanation, such as Zn(II) induced enzyme precipitation, inactivation caused by substoichiometric Zn(II) binding in the dodecamer, or inactivation due to multiple Zn(II) binding at the inhibitory sites in each subunit, cannot be ruled out. Nevertheless, it should be pointed out that attempts to detect enzyme precipitation have not been fruitful.

II. Identification of Substrate Induced Protein Conformational Changes in Glutamine Synthetase.

Based on protein fluorescence changes observed upon substrate binding, it is

assumed that these fluorescence changes are derived from protein conformational changes. In an attempt to identify the conformational changes described above, we have adenylylated the glutamine synthetase with a spin-labeled ATP analog, 2,2,6,6-tetramethylpiperidine-1-oxyl adenosine triphosphate (Tempo-ATP). The Tempo-adenylylated glutamine synthetase shows essentially identical catalytic properties, pH profile, and inhibitor susceptibility to those of the naturally adenylylated enzyme. The ESR spectrum of the covalently bound Tempo-AMP reveals that rotational correlation time of the nitroxyl moiety is 7.2×10^{-11} sec which is similar to 8.6×10^{-11} sec determined for the free Tempo-ATP. This observation indicates that the adenylylation site is located on the surface of the protein. Using the paramagnetic effect of Mn(II) on the ESR signal of the spin-labeled Tempo-GS, the distances from the nitroxyl moiety of the covalently bound Tempo-AMP to the structural divalent metal ion binding site (n_1 site) and the catalytic site (n_2 site) were determined when Mn(II) was used as divalent metal ion. In the absence of substrate, the distances from Mn(II) at the n_1 and n_2 sites to the nitroxyl moiety are 19 and 16 Å, respectively. These findings are in good agreement with data obtained with ^{13}C NMR and fluorescence energy transfer methods (Villafranca et al., Proc. Natl. Acad. Sci. U.S. 75, 1255-1259, 1978). Binding of substrate such as L-Glu causes a protein conformational change as indicated by the reduction of ~ 2 Å for the n_1 to Tempo-AMP distance and lengthening of ~ 2 Å for the n_2 to Tempo-AMP distance. Addition of ATP to the Tempo-GS·L-Glu complex increases the distances between n_1 and Tempo-AMP, and n_2 and Tempo-AMP by 4 and 3 Å, respectively.

III. Mechanistic Study of Actomyosin ATPase (for details see Annual Report of E. Eisenberg)

In contrast to the Lynn-Taylor model for actomyosin ATPase (Biochemistry 10, 4671, 1971) which suggests that ATP hydrolysis required the dissociation of ATP·actomyosin complex to ATP·myosin and actin, we have shown that myosin does not have to detach from actin during ATP hydrolysis (Stein et al., Proc. Natl. Acad. Sci. U.S. 78, 1346, 1981). In the present work, myosin subfragment-1 (A1) (S-1(A1)) was used to investigate the nature of the rate-limiting steps in the catalytic cycle of acto-S-1. The results obtained at 15°C, in very low ionic strength, suggest that a "six-state" kinetic model is necessary to explain the actomyosin ATPase. In this model, the rate-limiting step occurs after the cleavage of ATP, but precedes the rapid release of Pi. This rate-limiting step may play an important role in determining the velocity of muscle contraction.

Signifance to Biomedical Research

The overall objective is to gain a better understanding of how enzymes function with respect to their catalytic and regulatory properties, and to elucidate principles of interaction between effectors, regulators, and proteins. This knowledge is instrumental in controlling the function of a specific enzyme by designing an effector or enzyme suicide substrate. The study on glutamine synthetase provides some knowledge on how the protein conformation is varied due to substrate binding and how Zn(II) inactivates the enzyme.

Proposed Course of Research

(1) To further expand the cyclic cascade model to substrate cascade and more

complex enzyme cascade systems; and to further analyze the kinetic aspect of the cyclic cascade system.

(2) To study the mechanism of the cascade reactions which regulate the state of adenylylation for glutamine synthetase. We plan to study the protein-protein interaction between P_{II} protein and ATase, UTase, and UR, and ATase-glutamine synthetase interaction, by physical, chemical, and immunological methods, to test the validity of the bicyclic cascade model, and to study the role of the effectors in this cascade system. In addition, the phosphorylation cascade involving cAMP-dependent protein kinase and phosphoprotein phosphatase will be investigated using the fluorescent peptide synthesized in our laboratory.

(3) To further explore the physical, chemical, and immunological properties of unadenylylated and adenylylated glutamine synthetase. In particular, we will utilize the fast reaction techniques, NMR, ESR, and fluorescence polarization methods to elucidate the roles of effectors and to identify the intermediates in the catalytic cycle. Laser light scattering will be used to study protein-protein interactions.

Publications

Wright, D. E., Noiman, E. S., Chock, P. B., and Chau, V.: Novel fluorometric assay for adenosine 3',5'-monophosphate-dependent protein kinase and phsophoprotein phosphatase activities. Proc. Natl. Acad. Sci. U.S. 78: 6048-6050, 1981.

Mura, U., Chock, P. B., and Stadtman, E. R.: Allosteric regulation of the state of adenylylation of glutamine synthetase in permeabilized cell preparations of Escherichia coli. Study of monocyclic and bicyclic interconvertible enzyme cascades, in situ. J. Biol. Chem. 256: 13022-13029, 1981.

Stadtman, E. R., Chock, P. B., and Rhee, S. G.: Interconvertible enzyme cycles in cellular regulation. Curr. Top. Cell. Regul. 18: 79-94, 1981.

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Chau, V., Huang, C. Y., Chock, P. B., Wang, J. H., and Sharma, R. K.: Kinetic Studies of the Activation of Cyclic Nucleotide Phosphodiesterase by Ca^{2+} and Calmodulin. In Hidaka, H. (Ed.): Calmodulin and Calcium Receptors, in press, 1982.

Huang, C. Y., Rhee, S. G., and Chock, P. B.: Subunit cooperation and enzymatic catalysis. Ann. Rev. Biochem. 51: 937-971, 1982.

Rhee, S. G. and Chock, P. B.: Purification and Characterization of Uridylylated and Unuridylylated Forms of Regulatory Protein P_{II} Involved in the Glutamine Synthetase Regulation in E. coli. In M. J. Siciliano (Ed.): Isozymes: Current Topics Biological and Medical Research, New York, Alan R. Liss Pub. Co., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 00203-09 LB

PERIOD COVERED
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TITLE OF PROJECT (80 characters or less)

Cellular Regulation of Enzyme Levels

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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1.8	1.5	0.3

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have studied the regulation of intracellular enzyme level and protein turnover in Escherichia coli. We have proposed that the degradation of glutamine synthetase (GS) in E. coli is a two-step process involving inactivation followed by proteolysis (Levine, R.L. et al., (1981) Proc. Natl. Acad. Sci. 78, 2120-2124). We have characterized the properties of the inactivation reaction and several enzymic mixed function oxidation systems which catalyze GS inactivation (Oliver, C. and Stadtman, E. R., Annual Report 1980-1981) and we have demonstrated that enzymes other than GS are inactivated in a similar manner (Fucci, L., Oliver, C., and Stadtman, E. R., Annual Report 1981-1982). As an extension of these observations, studies have been initiated to examine the possible physiological role of bacterial enzyme inactivation by activated neutrophils which are capable of ingesting and killing bacteria. Other studies have been undertaken to isolate and purify an immunomodulator from activated macrophages. Techniques used in these studies have included pore gradient electrophoresis, isotopic labeling chromatographic techniques, enzyme assay, high performance liquid chromatography and tissue culture.

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Project Description

This project area consists of three parts. The first part concerns continuing studies on the mechanism of inactivation of GS and other key metabolic enzymes by mixed function oxidation systems and a detailed study of the rabbit liver microsomal cytochrome P450 inactivation systems. These studies have been undertaken in collaboration with Drs. L. Fucci and K. Nakamura of this laboratory. Major findings have been summarized in other sections of this Annual Report. Second, studies have been initiated to examine the possible physiological role of bacterial enzyme inactivation by activated neutrophils which are capable of ingesting and killing bacteria. Preliminary experiments with a promyelocytic cell line were carried out in collaboration with Drs. H. Weissbach and H. Fliss of the Roche Institute of Molecular Biology, Nutley, New Jersey. Finally, other studies have been undertaken in collaboration with Drs. Ira Green and John Schmidt of the National Institute of Allergy and Infectious Diseases to purify and characterize an immunomodulator which has IL-1 activity. This factor is a product which is elaborated and secreted by activated macrophages and specifically stimulates thymocytic and fibroblast growth and proliferation.

Major Findings

Neutrophil Studies: The process of bacterial enzyme inactivation may be functionally important in host defense against bacterial infection in higher organisms. Mature polymorphonuclear leukocytes (neutrophils) for example, are scavenger cells in higher organisms which are capable of ingesting and killing bacteria. These cells can be activated by a variety of compounds including bacterial chemotatic factor fmet-leu-phe or phorbol myristate acetate (PMA) to undergo a respiratory burst. This process is characterized by sharp changes in energy metabolism, by increased oxygen uptake and by production of several species of activated oxygen. Studies have shown that these activated oxygen species are diffusable and may be instrumental in target cell damage and eventual death (Babior, B. M. (1978) N. Engl. J. Med. 298, 659-668; Weiss, S. J (1980) J. Biol. Chem. 255, 9912-9917). Furthermore, neutrophils from patients with chronic granulomatous disease (CGD) exhibit normal phagocytosis but defective respiratory burst. These cells are unable to kill bacteria efficiently and patients with CGD often have severe recurring bouts of bacterial infection

We have previously presented evidence that H_2O_2 and Fe(II) are intermediates in the irreversible inactivation of GS and other enzymes mediated by mixed function oxidation systems. We have therefore initiated studies to determine if activated neutrophils are capable of inactivating E. coli GS and other key enzymes as a possible mechanism of bacterial cell killing. In order to avoid batch differences in pooled human neutrophils, we have used a human promyelocytic leukemia cell line designated HL-60 which can be stimulated to differentiate into neutrophils and can be activated to undergo a respiratory burst. Morphologically and biochemically, these differentiated cells are indistinguishable from normal peripheral neutrophils.

Early observations have indicated that high passage cells (passages 60-70) adapted to spinner cultures exhibit a slow growth rate with a doubling time of 48 hours and these cells undergo some spontaneous differentiation. These cells in fact exhibit some morphological features which are not typical of the myelocytic series. Because the HL-60 cell may represent a more primitive stem cell than the promyelocyte with a broader set of determiants, growth conditions may be very

important in defining a specific differentiation series.

Subsequently, low passage cells (passage 26) were obtained from Dr. T. Breitman (National Cancer Institute). These cells have been successfully differentiated and activated. Preliminary studies have indicated that growth is enhanced in RPMI 1640 with 20 mM Hepes buffer. Both 10% fetal calf serum and 2 mM glutamine are required for propagation but serum-free medium can be used for maintenance. For efficient growth, cultures must be seeded with 2-3 X 10^5 cells/ml. Cultures preconditioned in stationary flasks in 5% CO_2 will differentiate in spinner cultures.

Differentiation was initiated by growth in 1 μM retinoic acid. Differentiation was assessed by (1) morphology, (2) cessation of growth, and (3) generation of O_2, following PMA actiation which is quantitated by NBT or cytochrome C reduction. Cell-free extracts of HL-60 differntiated and control cells were prepared from cultures containing 1-4 X 10^6 cells/ml. The membrane and the supernatant fractions were assayed for endogenous GS activity and no activity was detected even after incubations longer than two hours.

When free E. coli GS was incubated with differentiated and control cells in the presence and absence of PMA, approximately 35-45% inactivation of GS was observed with the differentiated PMA activated HL-60 cells. No inactivation was observed in any other cultures. Similar results were obtained when whole E. coli was incubated with the same cells. Under these conditions, the HL-60 cells were treated with lysis buffer and E. coli GS was assayed in situ using CTAB permeabilization.

Although these results are exceedingly preliminary, they suggest that bacterial cell killing by neutrophils may involve inactivation of bacterial enzymes which leads to disruption of cellular metabolism and eventual cell death.

Proposed Course of Research

With the exception of growth studies which have been undertaken with low passage HL-60 cells, the early inactivation studies were carried out with high passage HL-60 cells. These studies will be repeated with low passage cells when conditions of growth, differentiation and activation have been adequately defined. Comparative studies are planned using normal human neutrophils, CGD neutrophils, and HL-60 cells which are chemically treated to block the respiratory burst and thus mimic CGD neutrophils.

If the neutrophil mediated inactivation of GS in intact E. coli can be verified, other key metabolic enzymes will be tested including those which have been found to be inactivated by enzymic mixed function oxidation systems (Fucci, L., Oliver, C., Stadtman, E. R., and Coon, M. J. (1982) Fed. Proc. 41, 5991). If general enzyme inactivation can be demonstrated, then studies will be undertaken to isolate and characterize the inactivation system(s) from neutrophils.

Major Findings

IL-1 Studies: In an effort to understand the mechanisms of connective tissue in inflammation, studies were undertaken to characterize a mixed lymphocyte mediated fibroblast proliferation factor by Drs. Ira Green and John Schmidt of the National Institute of Allergy and Infectious Diseases. They have previously demonstrated that a factor can be elaborated and secreted by macrophages in

response silica particles. These studies suggest that this factor may be involved in the chronic inflammation of silicosis, a fibrotic disease of lung which is characterized by fibroblast proliferation, collagen deposition, and loss of lung elasticity.

Preliminary characterization of a macrophage derived fibroblast proliferation factor indicated that it shared biochemical and functional properties with interleukin-1 (IL-1) a known (though not purified) macrophage derived immunomodulator. This factor stimulates thymocyte proliferation and is functionally important in cell mediated immunity and host defense in higher organisms. Initial studies were designed to separate the IL-1 and fibroblast proliferation activities. When macrophage culture supernatant fluids were analyzed by sephacryl S-200 or isoelectric focusing, identical activity profiles were obtained with the thymocyte proliferation assay (standard measure of IL-1 activity) and the fibroblast proliferation assay (standard measure of fibroblast proliferation activity). It should be pointed out that most tissues do not respond to IL-1 and there is no obvious reason why thymocytes and fibroblasts should both respond to IL-1. Similar molecular weights (~ 13,000) have been determined by gel filtration for the two activities and three charged species have been separated on isoelectric focusing, each having both thymocyte proliferation activity and fibroblast proliferation activity. The two activities could not be separated by SP-Sephadex chromatography, phenylsepharose chromatography, DEAE-chromatography, or by HPLC size exclusion chromatography. When subjected to heat denaturation, the two activities decayed at the same rate at 56°C. Although neither activity was sensitive to trypsin treatment, both activities were destroyed by chymotrypsin, S. griseus protease and subtilisin. In addition, the kinetics of inactivation of the two activities with chymotrypsin treatment were identical. These results suggest that fibroblast proliferation factor generated by macrophages is at least closely related and possibly identical with IL-1. The identity of the two molecules can best be established by purification, amino acid analysis, and sequence studies.

Proposed Course of Research

Characterization of human IL-1 has been hampered by three factors: (1) lack of an in vitro producer macrophage cell line, (2) extensive losses incurred by the use of conventional purification technqies, and (3) lack of a rapid sensitive in vitro assay.

A series of tumor derived human macrophage cell lines have been screened for IL-1 production without success. There is some evidence that the HL-60 cells noted above can be differentiated into macrophages and these cells will be tested in the hope of finding an in vitro culture system for IL-1 production. Currently, peripheral blood monocytes are obtained from normal leukophoresis donors and this represents a limited source of material.

Purification studies with high resolution high performance liquid chromatography using a Toya Soda SW 3000 size exclusion column have been highly successful. Analytic data indicates that IL-1 is eluted as a sharp peak with a molecular weight of approximately 14,000 and this single step yielded greater than 300-fold purification with 80-85% recovery of biological activity. Preparative isoelectric focusing has also been used and sharp resolution of three charged species has been reproducibly obtained. Method development studies using HPLC ion exchange and TMS reversed phase chromatography are planned. HPLC purification technology is expected to facilitate rapid purification without excessive loss of biological

activity. However, these studies have been severly hampered by lack of accessibity to HPLC instrumentation.

Preliminary results from both native and SDS gradient electrophoresis suggest subtle molecular weight differences between the two low pI species from isoelectric focusing (pI 5.1 and 5.4) and larger differences with the higher pI species (pI 6.8), as well as some quantitative differences in activity of these species. Although aggregation cannot be ruled out, these results suggest the possibility that processing by limited proteolysis may be involved in regulation of IL-1 activity. It should be pointed out that IL-1 is active at approximately 10^{-8}-10^{-10} M (based on estimates of protein in our most purified fractions and estimates of molecular weight, 14,000). We are currently purifying nanograms of IL-1 and for many analytical techniques, we are at the lower limits of protein detection. Therefore, biochemical characterization of this factor would be greatly facilitated by a rapid high yield purification procedure.

In addition, we should like to purify enough IL-1 to generate specific antibody. This would permit the development of a rapid sensitive *in vitro* radioimmunoassay which could at least partially replace the very tedious thymocyte proliferation bioassay.

It is likely that the interesting biology of IL-1 will involve its interaction with a specific receptor of responder target cells and the initiation of a cascade of events leading to growth and proliferation. In this context, we would like to investigate the possibility that IL-1 like some growth factors (PDGF, EDGF) interacts with a specific receptor containing a specific kinase as an integral part of the receptor. It might be possible to identify the IL-1 receptor by autophosphorylation of this kinase which could be activated by IL-1 receptor binding. Our understanding of IL-1 receptor interaction might elucidate some of the mechanism of chronic inflammation. Although purely speculative, mechanisms of chronic inflammation might involve continued stimulation of IL-1 production or lack of receptor turnover or both. Specific antibody for IL-1 and the IL-1 receptor would facilitate study of IL-1-receptor interaction and receptor antibody might function as a receptor blocker which could alleviate some of the deleterious effects of chronic inflammation or fibroblast proliferation in diseases such as lung silicosis.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00204-15 LB

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Protein Structure: Enzyme Action and Control

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4.2	3.4	0.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Research in this section consists of studies on the physical and chemical properties of proteins of biological interest and the roles of ligand binding and protein-protein interactions in enzyme catalysis and regulation. (1) Active-site ligand interactions with dodecameric glutamine synthetase from E. coli have been studied by spectrophotometric, binding, and calorimetric techniques using the diastereoisomers of L-met-S,R-sulfoximine separately and together. Conformational differences between unadenylylated and adenylylated enzyme forms on binding active-site ligands have been detected. (2) Reactivation of glutamine synthetase after auto-inactivation with L-met-S-sulfoximine, ATP, and Mn^{2+} (or Mg^{2+}) has been accomplished. The reactivation of the enzyme is first-order, dependent on the 3rd-4th power of $[H^+]$, and coincides with the release/subunit of 2 Mn^{2+}, L-met-S-sulfoximine-P, and ADP; increasing the pH from ≤ 5 to > 6 produces reinactivation. Inter- and intra-subunit bonding domains are markedly stabilized in the inactive enzyme complex. Submolecular, partially active and partially liganded oligomers containing 4,6,8, and 10 subunits have been isolated. (3) Equilibrium and kinetic studies of Me^{2+}-protein interactions (using chromogenic chelators and pH indicator dyes) have provided information on the structural and catalytic roles of Me^{2+}.

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Project Description

Objectives

(1) To study the physical and chemical properties of glutamine synthetase from Escherichia coli, particularly with respect to the correlation of the structure and catalytic function of this enzyme.

(2) To study conformational and stabilization changes of a protein macromolecule effected through the specific binding of small molecules and the relationship of such effects to enzyme catalysis and regulation.

(3) To study active-site ligand interactions with unadenylylated and adenylylated glutamine synthetases using spectrophotometric, binding, and calorimetric techniques in order to detect conformational differences between these enzyme forms.

(4) To determine the properties of the complex formed when ADP, L-methionine-S-sulfoximine phosphate, and two divalent cations are bound to active sites of E. coli glutamine synthetase, and to investigate the effects of these tightly bound active-site ligands on the tertiary and quaternary structure of the dodecameric enzyme.

(5) Kinetic and equilibrium studies of Mn^{2+}-glutamine synthetase interactions to determine structural and catalytic roles of divalent cations.

(6) Ultracentrifugal, calorimetric, and electrophoretic studies to determine macromolecular properties of biologically important proteins.

Major Findings

(1) Thermodynamic parameters for glutamine synthetase interactions with substrates and substrate analogs (Investigators: E. G. Gorman, A. Shrake, S. H. Neece, and A. Ginsburg).

Active-site ligand interactions with dodecmaeric glutamine synthetase from E. coli were studied by calorimetry using the resolved L-S- and L-R-diastereoisomers of the substrate analog L-methionine sulfoximine (E. G. Gorman and A. Ginsburg, in press). Measurements were made at 30°C and pH 7.1 in buffers (containing 100 mM KCl and 1 mM $MnCl_2$) with different heats of protonation, allowing calculation of the eq of H^+ either taken up or released for each binding reaction and correction of the enthalpy of binding for buffer proton effects (ΔH). For binding either the S- or R-isomer of L-methionine sulfoximine to the unadenylylated, manganese enzyme, $\Delta H = -12 \pm 1$ kcal/mol of enzyme subunit with a small net proton release ($\leq$ 0.2 eq of H^+/mol) implicated in subunit interactions. The corresponding thermal titration data also give other thermodynamic parameters at 303°K (standard state for hydrogen ion activity at $10^{-7.1}$ M): $\Delta G' = -6.06$ and -4.42 kcal/mol of subunit for binding S- and R-isomers, respectively, and $\Delta S \simeq -23$ cal/(deg·mol of enzyme subunit) for binding either isomer. Furthermore, the reaction heat for the simultaneous binding of the S- and R-isomers to unadenylylated enzyme (with a concomitant net uptake of 0.7 eq of H^+/subunit) is more exothermic than with either isomer alone, suggesting the occurrence of subunit interactions in this case also. For the binding of the S- and R-isomers of L-methionine sulfoximine to fully adenylylated, manganese glutamine synthetase, $\Delta H = -28$ and -13 kcal/mol of enzyme subunit with an accompanying uptake of 1.7 eq of H^+ and release of ~ 0.3 eq of H^+ per subunit, respectively. Thus, ionizable

protein groups are perturbed by active-site ligand binding to the adenylylated enzyme, contributing to apparent binding enthalpies. Enthalpy changes and proton effects observed on binding L-methionine-S-sulfoximine or L-glutamine to unadenylylated and adenylylated, manganese enzymes reveal conformational differences between these enzyme forms that may be subtle but catalytically significant, involving mainly pK perturbations of ionizable protein groups without major structural alterations.

Table I summarizes the above ΔH values and proton effects for unadenylylated ($Mn \cdot GS_1^-$) and fully adenylylated ($Mn \cdot GS_{12}^{--}$) enzymes binding active-site ligands at pH 7.1 (30°C). All values are given per mole of enzyme subunit for complete saturation with ligand; ΔH values (corrected for buffer proton effects) are in kcal/mol with an estimated error of ± 1.2 kcal/mol and proton values are in eq of H^+/mol of uptake (+) or release (-) in the binding reactions.

Table I. Binding Enthalpies and Proton Effects

Active-site ligand added to enzyme at pH 7.1 (30°C)	$Mn \cdot GS_1^-$		$Mn \cdot GS_{12}^{--}$	
	ΔH	H^+ eq	ΔH	H^+ eq
L-Met-S-sulfoximine	-12.5	-0.1	-28.2	+1.7
L-Met-R-sulfoximine	-11.5	0	-12.8	~ -0.3
1:1 Mixture of S- and R-isomers	-18.8	+0.7	-25.2	+1.2
L-Glutamine	- 9.7[a]	0[a]	- 6.1	-0.3

[a]Values are from Shrake, A., Powers, D. M., and Ginsburg, A. (1977) Biochemistry 16, 4372-4381.

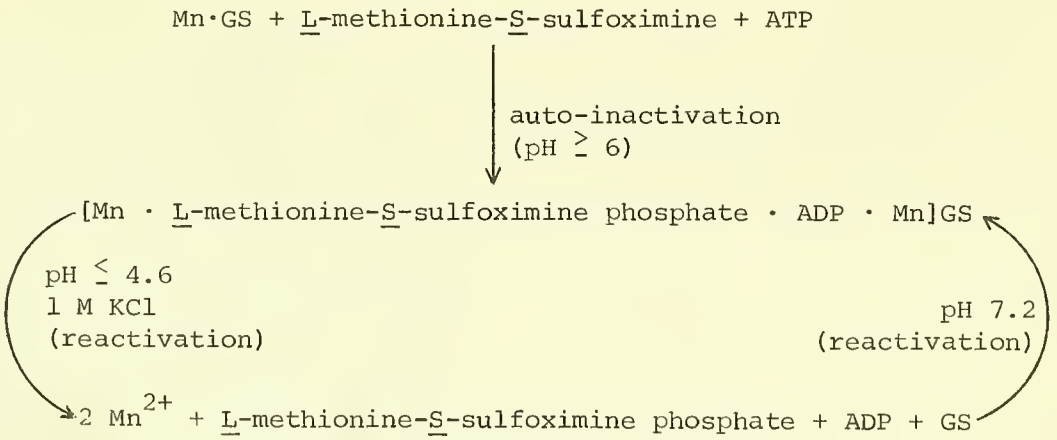
The proton effects observed with the equimolar mixture of S- and R-isomers of L-methionine sulfoximine as well as the increased affinity of the enzyme for R-isomer produced by partial saturation of glutamine synthetase with S-isomer (Shrake et al., 1982), are indicative of subunit interactions. In the equimolar mixture of S- and R-isomers, both diastereoisomers compete for the same enzyme subunit site with about the same affinity in binding (A. Shrake, E.J. Whitley, Jr., and A. Ginsburg, J. Biol. Chem. 255, 581-589, 1980). If, at a saturating level of ligands, ~ 50% of the enzyme sites are filled with each isomer, the proton uptake with the equimolar mixture of S- and R-isomers should equal ~ one-half of the sum of the proton effects observed with the resolved isomers. This is not the case with either the unadenylylated or adenylylated enzymes (Table I). Furthermore, ΔH values for binding the mixture of S- and R-isomers are 5 to 7 kcal/mol more exothermic than values calculated from the sum of one-half ΔH for S-isomer binding plus one-half ΔH for R-isomer binding. The more negative ΔH values for binding the equimolar mixture of S- and R-isomers could correlate with the partial protonation of protein groups perturbed through subunit interactions.

As an extension of the above studies, we have more recently measured the heats of binding the resolved S- and R-isomers of L-methionine sulfoximine to the

Mn·AMP-PNP enzyme complex. The ATP analog adenylylimidodiphosphate (AMP-PNP) was used so that phosphorylation of the S-isomer could not occur. In addition, for binding AMP-PNP to the enzyme, $\Delta H \leq +1.8$ kcal/mol was measured at 30°C. There is marked synergism between the binding of L-methionine-S-sulfoximine and AMP-PNP and between the binding of L-glutamate and ATP. To obtain $\Delta G'$ values, fluorometric titrations, UV spectral titrations, or equilibrium binding measurements are being performed. Eventually, thermodynamic parameters for glutamine synthetase interactions with combinations of active-site ligands will be obtained.

(2) Reactivation of glutamine synthetase from E. coli after autoinactivation with L-methionine-S-sulfoximine, ATP, and Mn^{2+} (Investigators: M. R. Maurizi, and A. Ginsburg).

E. coli glutamine synthetase auto-inactivated with L-methionine-S-sulfoximine and ATP can be completely reactivated at pH 3.5-4.6 in 1 M KCl and 0.4 M $(NH_4)_2SO_4$. Both unadenylylated and adenylylated magnesium and manganese enzymes can be reactivated. Reactivation of fully inactivated enzyme is first order ($t_{1/2} \simeq 2$ min at pH 4.1 and 37°C) and coincides with the stoichiometric release of 0.95 ± 0.05 eq each of L-methionine-S-sulfoximine phosphate and ADP and 2.0 ± 0.2 eq of Mn^{2+} from each subunit. The rate of reactivation increases with decreasing pH and is proportional to the 3rd or 4th power of the hydrogen ion activity; the protonation of 3-4 carboxylic acid groups/subunit therefore may be required to disrupt the enzyme complex. Reactivation rate also increases with increasing KCl concentrations and temperature, with an Arrhenius activation energy of ~ 26 kcal/mol, sugtesting that some protein structural perturbation is required to disrupt the complex. Upon neutralization of reactivation solutions, the ligands and metal ions recombine with the enzyme resulting in its complete reinactivation. Thus, inactivation of glutamine synthetase at neutral pH is due to the extremely tight, but reversible, binding of L-methionine-S-sulfoximine phosphate, ADP, and Mn^{2+} ($K_A' > 10^{12}$ M^{-1} for ADP) to the enzyme. Under certain conditions, ATP or ADP can partially inhibit inactivation. Excess ADP inhibits reinactivation by L-methionine-S-sulfoximine phosphate, and ATP has a complex effect on the rate and extent of the autoinactivation reaction.



Scheme 1

Scheme 1 summarizes the autoinactivation reaction and conditions for dissociation (reactivation) and reassociation (reinactivation) of active-site ligands

in reversible binding processes. When the pH of the reactivation solution was raised above pH 6, L-methionine-S-sulfoximine phosphate, ADP, and Mn^{2+} bound rapidly to the enzyme, resulting in complete reinactivation of the enzyme (Scheme 1). Reinactivation involved the rebinding of free components of the inactive complex since [^{14}C]ADP released from the inactive complex was freely exchangeable with exogeneous ADP added during reinactivation and chelation of Mn^{2+} with EDTA completely inhibited reinactivation. Reinactivation was rapid even when the concentration of glutamine synthetase subunit, ADP, and L-methionine sulfoximine was diluted to 0.2 μM in the presence of 1 mM $MnCl_2$. The kinetics of the reinactivation with excess Mn^{2+} added appeared to be second order (~ 10^6 M^{-1} s^{-1} at 25°C) which suggests that the reassociation of only one ligand is required for inactivation. This conclusion was confirmed by removing ADP prior to neutralization and finding that reinactivation proceeded rapidly with only L-methionine-S-sulfoximine phosphate and Mn^{2+} present. Estimated affinity constants for the active site ligands of the inactive enzyme complex are $K_A' > 10^9$ M^{-1} for Mn^{2+} (J. Hunt and A. Ginsburg, J. Biol. Chem. 255, 590-594, 1980) and $K_A' > 10^{12}$ M^{-1} for ADP (M. R. Maurizi and A. Ginsburg, J. Biol. Chem. 257, 4271-4278, 1982).

In summary, the very tight, synergistic binding of four components containing a transition state analog to the active site of each subunit dodecameric glutamine synthetase from E. coli has been demonstrated. The conditions for reversibly binding these ligands give some insight into the role that protein structure has during catalysis.

The octameric glutamine synthetase from bovine brain has been purified on a small scale. Reactivation (under the conditions used for the E. coli enzyme) after autoinactivation with L-methionine sulfoximine, ATP, and Mn^{2+} (or Mg^{2+} was observed transiently and the yields were poor. The brain enzyme will be purified in larger quantities and conditions for stabilizing its activity will be further explored. However, a complete loss in activity during the autoinactivation reaction corresponded to the tight binding of ligands to only half of the subunits of the octamer. Nucleotide effects were similar to those observed with E. coli enzyme.

(3) Active site ligand stabilization of quaternary structures of glutamine synthetase from E. coli (Investigators: M. R. Maurizi and A. Ginsburg).

Autoinactivated E. coli glutamine synthetase contains 1 eq each of L-methionine-S-sulfoxmine phosphate and ADP and 2 eq of Mn^{2+} tightly bound to the active site of each subunit of the dodecameric enzyme (M. R. Maurizi and A. Ginsburg, J. Biol. Chem. 257, 4271-4278, 1982). Complete dissociation and unfolding in 6 M guanidine HCl at pH 7.2 and 37°C requires > 4 hours for the autoinactivated enzyme complex (< 1 min for uncomplexed enzyme). Release of ligands and dissociation and unfolding of the protein occur in parallel but follow non-first order kinetics, suggesting stable intermediates and multiple pathways for the dissociation reactions. Treatment of partially inactivated glutamine synthetase (2-6 autoinactivated subunits/dodecamer) with EDTA and dithiobisnitrobenzoic acid (DTNB) at pH 8 modifies ~ 2 of the 4 sulfhydryl groups of unliganded subunits and causes dissociation of the enzyme to stable oligomeric intermediates with 4, 6, 8, and 10 subunits. Other sulfhydryl reagents (paramercuriphenyl sulfonate, iodoacetamide) can be substituted for DTNB. The distribution of oligomers is dependent on the percent inactive complex on the enzyme prior to treatment with EDTA and sulfhydryl reagent, with tetramers and hexamers predominating at low extents of inactivation

and larger oligomers being favored at higher extents of inactivation. With > 70% inactivated enzyme, no dissociation occurs under these conditions. Oligomers are composed of approximately equal numbers of uncomplexed subunits and autoinactivated subunits, as shown by quantitating radioactive ADP bound to isolated tetramers and by enzymatic assay of reconstituted tetramers and hexamers (Table II). The large inactive aggregate ($M_r > 10^6$) formed (Table II) does not contain the inactivating ligands; this amorphous aggregate appears to result from nonspecific reassociation of unliganded monomer or dimer. Isolated tetramers (M_r = 200,000; $S_{20,w}$ = 9.5 S) retain sufficient native structure to express significant enzymatic activity, since light scattering measurements of tetramers in assay mixture without 2-mercaptoethanol showed that the tetramer did not reassociate. Upon addition of 2-mercaptoethanol to remove the thionitrobenzoate groups, there was a rapid, 3-fold increase in light scattering, corresponding to reassociation of tetramers to dodecamers and a 5-fold increase in activity (Table II). Electron micrographs of oligomers (R. H. Haschemeyer, J. S. Wall, J. Hainfeld, and M. R. Maurizi, J. Biol. Chem. 257, 7252-7253, 1982) show that dissociation of partially liganded dodecamers occurs by cleavage of intra-ring subunit contacts across both hexagonal rings producing structures that contain equal numbers of superimposed subunits from the two hexagonal rings of the original dodecamer. This appearance of the oligomers strongly suggests that dissociation of glutamine synthetase can occur by the stepwise release of pairs of isologously bonded subunits from the opposite rings and also suggests that the active-site ligand increases the stability of the heterologous bonds between subunits in the same ring. Further studies, now underway, will be needed to determine the distribution of inactive subunits within partially inactive glutamine synthetase and within the various oligomers and to confirm the above conclusion.

Preliminary studies directed toward addressing the above and related concerns are of two kinds (a) efforts to obtain reagents for labeling either the uncomplexed subunits or the inactive complex subunits in mixed oligomers, and (b) efforts to devise analytical methods for the separation of hybrid dodecamers consisting of chemically distinguishable subunits of glutamine synthetase. For part (a) above, two different reagents have been sought. Analogs of ATP modified at the C-8 position with chemically reactive groups would provide a method of specifically labeling the active-site of glutamine synthetase with reporter groups that can be visualized by electron microscopy. A collaborative study with J. Hainfeld and J. Wall at Brookhaven National Laboratory has been initiated. A heavy-metal containing reagent that can react with the amino- or thiol-ATP derivatives is being prepared. Secondly, antibodies against the sulfhydryl reagent, dithiobisnitrobenzoic acid, are being raised. These antibodies should be able to localize the uncomplexed subunits of glutamine synthetase oligomers since only those subunits have sulfhydryl groups available for reaction with DTNB. For part (b) above, efforts have been concentrated on developing an affinity electrophoresis procedure for separating glutamine synthetase with various states of adenylylation. Initial results with ADP-derivatized polyacrylamide gels run in the presence of Mn^{2+} and arsenate at pH 7.8 show a substantial separation of fully adenylylated from fully unadenylylated enzyme. Methods are now being sought to optimize conditions for separation to produce the sharp nondiffused protein bands necessary to separate enzyme with intermediate states of adenylylation. Other chemical and physical methods to distinguish glutamine synthetase subunits, such as acylation of amino groups to produce charge isomers, are being examined.

Table II. Enzymatic Activity of Oligomers

Oligomers were prepared by treating 15% inactivated enzyme with EDTA and DTNB. After inactivation of the enzyme with limiting L-methionine-S-sulfoximine, the enzyme was treated with EDTA at pH 7.2 followed by an incubation for 30 minutes at pH 8 (37°C) with excess DTNB. Oligomers were separated by high pressure gel filtration and various fractions were assayed in the standard γ-glutamyltransferase assay solution with and without addition of 30 mM 2-mercaptoethanol.

Fraction from gel filtration	Predominant oligomer	Glutamine synthetase activity	
		No addition	2-Mercapto-ethanol added
		units/mg	
2	Aggregate	0.40	0.92
5	Dodecamer/decamer	26	35
6	Octamer	21	29
7	Hexamer	16	39
9	Tetramer	8	40
	Untreated, native enzyme	99	99

(4) Active-site ligand effects on kinetics of Mn^{2+} dissociation from E. coli glutamine synthetase (Investigators: J. B. Hunt, P. M. Grant, and A. Ginsburg).

Dodecameric glutamine synthetase from E. coli has a catalytic site and 2 essential Me^{2+} sites (n_1 and n_2) per subunit. Mn^{2+} binds to n_1 sites of the enzyme in a low- and high-affinity conformation, GS^R and GS^T, respectively (J. Hunt and A. Ginsburg, Biochemistry 20, 2226, 1981). However, active-site ligands stabilize additional conformations of $Mn{\cdot}GS^T$, which have decreased rates of Mn^{2+} dissociation. Using the chromogenic Me^{2+}-chelator BAPTA (R. Y. Tsien, Biochemistry 19, 2396, 1980) at pH 7.2 and 25°C, half-times for the dissociation of Mn^{2+} from n_1 sites ($Mn{\cdot}GS^T \rightarrow GS^T + Mn^{2+}$) were measured by stopped-flow: without effectors, $t_{1/2} \simeq 0.39$ s; with 150 mM L-glu, $t_{1/2} \simeq 1.4$ s; with 1 mM of the L-glu analog L-met-SR-sulfoximine, $t_{1/2} \simeq 2.1$ s. Displacing Mn^{2+} bound at n_1 sites by Ca^{2+} in the presence of the chelator xylenol orange gave $t_{1/2} \simeq 0.25$ s; $t_{1/2} \simeq 2.5$ s when 1 mM L-methionine sulfoximine was added. The half-time of the overall relaxation ($Mn{\cdot}GS^T \rightarrow GS^R$), for which the conformational change $GS^T \rightarrow GS^R$ is the slow step, is ~ 44 s at 25°C with EDTA present and is increased ~ 2-fold by L-glutamine and ~ 5-fold by L-methionine sulfoximine. Without or with active-site ligands present, the activation energy is 32 kcal/mol for $Mn{\cdot}GS^T \rightarrow GS^R$. There is kinetic evidence that chelators bind to protein-bound Mn^{2+}, facilitating Mn^{2+} release from the protein. However, the retardation of Mn^{2+} release from n_1 sites by active-site ligands is independent of the Me^{2+}-chelator present.

The evidence for the formation of ternary enzyme-Mn^{2+}-chelator complexes, with the chelator serving to aid the removal of Mn^{2+} from the enzyme is as follows: (a) The identity of the chelator influences the rate of $MnGS^T \rightarrow GS^T$. (b) The rates for $MnGS^T \rightarrow GS^T$ are faster than expected if complete dissociation of

Mn^{2+} from $MnGS^T$ precedes the reaction of Mn^{2+} with the chromogenic chelator, i.e., from the fastest rate that Mn^{2+} can react with GS^T of 6×10^6 sec^{-1} M^{-1}, which is the rate constant for substitution of inner sphere water of Mn^{2+} (M. Eigen), and the affinity constants of GS^T for Mn^{2+} of 2×10^6 M^{-1} and 6×10^7 M^{-1} in the absence and presence of L-methionine sulfoximine, respectively, corresponding minimum $t_{1/2}$ values from off rates are 0.23 sec and 6.9 sec. (c) Amplitudes of absorance changes in the stopped-flow instrument are much larger than expected from the relative affinities of the chromogenic chelator and GS^T for Mn^{2+} if Mn^{2+} must dissociate completely from $MnGS^T$ before binding chelator.

(5) Investigation of Zn^{2+} domains in aspartate transcarbamoylase (ATCase) from E. coli (Investigators: J. B. Hunt, S. H. Neece, A. Ginsburg, and H. K. Schachman).

Preliminary studies have indicated that we can use a high-affinity ($K'_A > 10^7$ M^{-1}) chromogenic chelator 4-(2-pyridyl azo) resorcinol (PAR) for binding Zn^{2+} released from ATCase. PAR forms a 2:1 dye:Zn^{2+} complex and has a very high absorption change ($\Delta E_{500\ nm} = 7.4 \times 10^4$ M^{-1} cm^{-1}) at 500 nm upon complexing Zn^{2+}. ATCase contains 4 sulfhydryl groups per regulatory chain and all of these are involved in the very tight tetrahedral binding of Zn^{2+} near catalytic and regulatory chain contact regions in intact ATCase which is composed of 6 catalytic (c) and 6 regulatory (r) chains (S. Subramani and H. K. Schachman, J. Biol. Chem. 256, 1255, 1981; Monoco et al., Proc. Natl. Acad. Sci. U.S. 75, 5776, 1978). In the presence of PAR, we can react the -SH groups of the regulatory chains with p-chloromercuricphenyl sulfonate (PMPS) and kinetically follow the Zn^{2+} release by the absorption change at 500 nm. This information will be correlated with previous results on sulfhydryl reactivity in the absence and presence of the bisubstrate analog N-(phosphonacetyl)-L-aspartate (PALA).

Significance to Biomedical Research

The regulation and control of enzymic activities in vivo is of fundamental importance in cellular metabolism. Through studies in vitro, these processes can be understood more fully. The studies of structural changes that can be induced in a protein macromolecule are important in understanding cellular processes on a molecular basis.

Proposed Course of Research

(1) To study conformational and stabilization changes of a protein macromolecule effected through the specific binding of small molecules and the relationship of such effects to enzyme catalysis and regulation. Ultracentrifugation, calorimetry, spectral, viscometry, fluorescence, equilibrium binding, pH, electrophoretic, and kinetic techniques will be used.

(2) To study mutual interactions of divalent cations, substrates (or substrate analogs), and inhibitors with glutamine synthetase from E. coli. Covalent modification will be used to obtain information on the topography of divalent cation, substrate, and inhibitor sites. Protein conformational changes produced by the binding of substrates and substrate analogs to glutamine synthetase will be investigated further.

(3) To characterize the thermal transition of glutamine synthetase more fully in terms of kinetic parameters. Also, the nature of the temperature-in-

duced unfolding reaction (local vs temperature-induced macromolecular shape changes) will be investigated by viscometry, ultracentrifugation, and CD techniques.

(4) Kinetic and equilibrium studies of the interactions between metal ions and glutamine synthetase will be continued. For this purpose, metal ion chromographic chelators and pH indicator dyes will be used in the absence and presence of active-site ligands.

(5) Glutamine synthetase from bovine brain will be purified in order to determine the role of divalent cations in the catalytic mechanism of the mammalian enzyme. After auto-inactivation of the mammalian enzyme with L-methionine-S-sulfoximine, ATP, and divalent cations, the stoichiometry of Me^{2+} binding will be determined and conditions for reactivation of the enzyme will be explored. The effects of inactive complex formation on intra- and inter-subunit bonding domains of this octameric enzyme will be investigated also.

(6) Studies on the removal of Zn^{2+} from aspartate transcarbamoylase (ATCase) from E. coli will be performed in collaboration with H. K. Schachman (University of California, Berkeley, CA). Since sulfhydryl groups at Zn^{2+} sites on regulatory chains are expected to be highly reactive in the absence of Me^{2+} ions, immediate Me^{2+} ion substitution of Zn^{2+} sites may be necessary to preserve the protein structure. Because intact ATCase has an enormous affinity for Zn^{2+}, it may be possible to kinetically follow the 'correct' folding of regulatory chains for -SH group chelation to Zn^{2+} and the dependence on the presence of catalytic chains for forming catalytic and regulatory chain contacts using a Zn^{2+}-chromogenic chelator. In addition, the stability of the Zn^{2+} binding domain can be examined now in intact ATCase (c_6r_6) as well as the influence of active-site and allosteric ligands on Zn^{2+} binding domains

Publications

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Shrake, A., Ginsburg, A., Wedler, F.C., and Sugiyama, Y.: On the binding of L-S- and L-R-diastereoisomers of the substrate analog L-methionine sulfoximine to glutamine synthetase from Escherichia coli. J. Biol. Chem. 257, in press.

Gorman, E.G. and Ginsburg, A.: Binding enthalpies for glutamine synthetase interactions with L-S- and L-R-diastereoisomers of the substrate analog L-methionine sulfoximine. J. Biol. Chem. 257, in press.

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PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Occurrence and Biochemical Roles of Selenium in Selenoproteins and Seleno-tRNAs

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

None

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.3	2	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Amino acid compositions of thiolases (seleno-thiolase and non-selenothiolase) were found to be similar. The native enzyme 158,000 to 160,000 Mr contains 16 cysteine residues but only 4 of these were titratable with DTNB or were detected as [14-C]carboxymethyl cysteine in hydrolysates of native protein alkylated with [14-C]iodoacetic acid. [75-Se]thiolase isolated from Clostridium kluyveri contained selenium in the form of [75-Se]selenomethionine. This seleno amino acid was identified by chromatography on an amino acid analyzer, by TLC analysis and by its ability to be converted to a product indistinguishable from Se-adenosylselenomethionine by S-adenosyl methionine synthetase. Inhibition of growth of Clostridium sticklandii by the antibiotic monensin also resulted in inhibition of the synthesis of [75-Se]selenoprotein A. Radioactivity that initially copurified with the tRNA fraction from the monensin-treated cells was unstable suggesting that a precusor to the seleno-tRNAs normally formed had accumulated in the presence of monensin.

Project Description

1. Anaerobic metabolism of certain amino acids with special references to the role of selenium, molybdenum, quinones, flavins and non-heme iron proteins in the electron transfer and phosphorylation reactions involved.

   a. Structure and function of the selenoprotein component of glycine reductase and its interaction with other protein components of the enzyme complex. Mode of biosynthesis of the selenium-containing moiety; a selenocysteine residue of the selenoprotein.

   b. Purification and characterization of Fraction C component of glycine reductase complex. Preparation of substrate amounts of selenoprotein A and carbonyl protein B in homogeneous form. Studies on the mechanism of glycine reduction and the coupled phosphorylation process by glycine reductase reconstituted from pure protein components A, B and C.

2. Isolation and characterization of other seleno-enzymes and selenium containing tRNAs.

   a. Formate dehydrogenases of Methanococcus vannielii: Comparison of the selenium-dependent and selenium-independent formate dehydrogenases and effect on catalytic activity of tungsten substitution for molybdenum.

   b. Characterization and biochemical role of seleno-thiolase produced by Clostridium kluyveri and seleno-nicotinic acid hydroxylase produced by Clostridium barkeri.

   c. Mechanism of specific modification of tRNAs by selenium and biochemical roles of seleno tRNAs.

3. Methane biosynthesis from formate and acetate and the roles of vitamin B-12 and 5-deazaflavin in the process.

Major Findings

(1) Studies of the effects of various antibiotics on the incorporation of ^{75}Se into proteins of Clostridium sticklandii were extended to include monensin, a monovalent cation ionophore. Levels of monensin that prevent cell multiplication of C. sticklandii (10^{-7} to 10^{-6}M) inhibited the synthesis of [^{75}Se]selenoprotein A of glycine reductase and also allowed very little formation of [^{75}Se]-tRNAs. ^{75}Se that initially copurified with the tRNA fraction was separated subsequently by phenol extraction suggesting the presence of a labile precursor. In contrast, [^{75}Se]tRNAs are formed in the presence of other antibiotics such as chloramphenicol and rifampsin even when cell multiplication and selenoprotein A biosynthesis are inhibited. Synthesis of two unidentified ^{75}Se-labeled proteins in the presence of monensin, as judged by the detection of [^{75}Se]polypeptides in slab gel electrophoretic profiles, apparently occurred. The possibility that one of these is a selenothiolase will be further investigated and the monensin-treated cells will be studied in greater detail to see if they will prove useful in characterizing the biosynthetic pathways leading to formation of Se-modified bases in tRNAs, selenocysteine in selenoprotein A or selenomethionine in thiolase.

(2) Amino acid compositions of thiolases isolated in homogeneous form from Clostridium kluyveri were determined. These were similar for the selenium-

containing thiolase and the so-called non-selenothiolase except for the presence of selenomethionine in the latter. Both thiolases contain two types of subunits which are detectable as two barely separable protein bands (ca 40,000 M_r) in SDS gels. The 160,000 M_r native proteins thus appear to be made up of four ~40,000 M_r subunits. However, selenium present in the selenothiolase is equivalent to only about one equivalent per mole of native protein and this occurs in the form of a selenomethionine residue. This was identified in acid hydrolysates of the native protein by amino acid analyzer and TLC analyses. Furthermore, the ^{75}Se-labeled amino acid, after isolation on the amino acid analyzer column, was converted to an ^{75}Se-labeled basic product, by reaction with ATP and S-adenosylmethionine synthetase, that was indistinguishable from authentic Se-adenosylselenomethionine. This is the first example of the occurrence of selenomethionine in an enzyme produced in the presence of a high sulfur to selenium ratio and therefore presumably incorporated as a specific moiety rather than as a non-specific substitution for methionine.

Growing cultures of C. kluyveri actively degrade ^{75}Se-selenomethionine and produce volatile ^{75}Se (either $CH_3{}^{75}SeH$ and/or $H_2{}^{75}Se$). Either [^{75}Se]selenomethionine or [^{75}Se]selenite serves as source of ^{75}Se for synthesis of labeled proteins. Growth is unaffected by inclusion of methionine in the culture medium (up to 5 mM levels) and, therefore selenothiolases isolated from cells grown in the presence of 0.5 μM [^{75}Se]selenomethionine and 1000-10,000 molar excesses of the sulfur analog will be analyzed to see if the selenomethionine content of the enzyme varies as a function of these conditions.

Selenothiolase contains 4 equivalents of readily alkylatable cysteine per mole and 12 additional cysteine residues (detectable as cysteic acid after oxidation) or a total of 16 cysteine residues per 160,000. Thiolases from other sources are similar in this respect.

Very little [^{75}Se]tRNA was found in three different batches of C. kluyveri ^{75}Se-labeled cells that were examined. In contrast, both C. sticklandii and Methanococcus vannielii contain significant levels of tRNA species specifically modified with selenium.

Proposed Course of Research

(1) Glycine reductase: Further characterization of the selenoprotein A component as a means of elucidation of its biochemical role in the catalytic reaction. The proximity of the selenocysteine residue and the highly reactive two cysteine residues in the polypeptide will be investigated in more detail. From the tyrosine and phenylalanine content of the protein 5 (or possibly 6) chymotryptic peptides are expected. In earlier studies selenoprotein A that had been reduced and alkylated with [^{14}C]iodoacetic acid was cleaved with chymotrypsin. Two radioactive peptides were separated from the digest on the basis of their differing net charges. Analysis of acid hydrolysates of these peptides showed that one contained both S-carboxymethylcysteine and Se-carboxymethylselenocysteine whereas the other contained only S-carboxymethylcysteine. These results indicate that the two cysteine residues are separated by at least one chymotryptic cleavage site. To improve the yields of these labeled peptides and to facilitate detection of the selenocysteine residue similar experiments will be performed using ^{75}Se-labeled protein A derivatized with ^{14}C-labeled alkyl groups and separation of peptides under anaerobic conditions by high performance liquid chromatography. The amino

acid compositions of the pure peptides and sequences will then be investigated. A glycopeptide (or peptides) derived from the enzymic digests will be recovered, if possible, and subjected to sugar analysis in order to complete the identification of the glycosyl groups attached to selenoprotein A. So far only glucose and mannose have been identified.

(2) Seleno-thiolase: The stoichiometry of selenomethionine in thiolase preparations derived from Clostridium kluyveri under a variety of growth conditions (e.g. varying selenomethionine to methionine ratios in the growth medium) will be determined. The mode of attachment of this seleno amino acid to the enzyme and its precise location will be investigated as well as its mechanism of synthesis from selenite, etc. Preliminary evidence that Clostridium sticklandii contains a seleno-thiolase will be followed up.

(3) Seleno hydrogenase: The hydrogenases in C. kluyveri and in C. sticklandii will be examined from ^{75}Se-labeled bacterial cells (in cooperation with S. Yamazaki) to see if the enzyme from these sources also is a selenoprotein.

Honors

Chairman of 1982 ASBC Travel Awards Committee for Australian Biochemical Congress, Perth, Australia.

Vice Chairman, U.S. National Committee to International Union of Biochemistry, 1981-82.

Member, NIH Alumni Association Committee, 1981 - present.

Member, Scholars Advisory Panel, Fogarty Scholars-In-Residence Program, 1980 - June 30, 1982.

Member, Promotion Review Committee, National Institute of Mental Health, 1981 - present.

Elected to membership in National Academy of Arts and Sciences, May 1982.

Publications

Stadtman, T.C.: Selenoenzymes in "Structural and Functional Aspects of Enzyme Catalysis", 32 Colloquium-Mosbach, pages 96-103, Ed. Eggerer and Huber. Springer-Verlag, Berlin, October 1981.

Ching, Wei-Mei, and Stadtman, T.C.: Selenium containing $tRNA^{Glu}$ from Clostridium sticklandii: Correlation of aminoacylation with selenium content. Proc. Natl. Acad. Sci. USA 79, 374-377 (1982).

Baker, John J., and Stadtman, T.C.: Amino Mutases in "B_{12}", Vol. 2, pp. 203-232 (1982) Ed. David Dolphin, John Wiley & Sons, Inc. New York.

Yamazaki, Shigeko, Tsai, L., and Stadtman, T.C.: Analogues of 8-hydroxy-5-deazaflavin Cofactor: Relative activity as substrates for 8-hydroxy-5-deazaflavin-dependent $NADP^+$ reductase from Methanococcus vannielii. Biochem. 21, 934-939 (1982).

Hartmanis, Maris G.N., and Stadtman, T.C.: Isolation of a selenium-containing thiolase from Clostridium kluyveri: Identification of the selenium moiety as selenomethionine. Proc. Natl. Acad. Sci. USA (in press) (August 1982).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00206-23 LB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Stereochemical Studies of Enzymatic Reactions (5-Deazaflavin)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.: Lin Tsai Research Chemist LB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Intermediary Metabolism and Bioenergetics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1	0.3

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

(1) Syntheses of three highly simplified analogues of 8-hydroxy-5-deazaflavin cofactor.
(2) Synthesis of a novel seleno-nucleoside, 5-methylaminomethyl-2-selenonuridine.

PHS-6040
(Rev. 2-81)

Project Description (1)

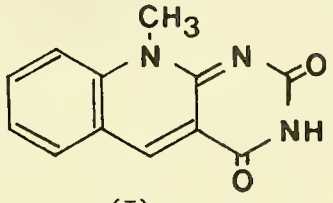
Objectives: 8-Hydroxy-5-deazaflavin-dependent $NADP^+$ reductase catalyzes the reaction:

Dihydro-8-hydroxy 5-deazaflavin anion + $NADP^+$ $\rightleftharpoons$ 8-Hydroxy 5-deazaflavin + NADPH

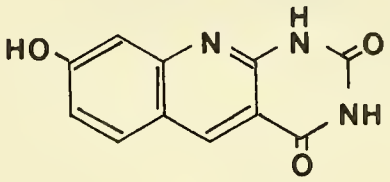
(A) To elucidate further the substrate structure-reactivity relationships for this enzyme, highly simplified analogues of the natural cofactor were examined. (B) In order to develop methodology for the determination of the stereochemical course of the enzymic reaction, chemical reactions of the dihydro compound were studied.

Major Findings (1)

(A) Since the parent heteroaromatic system (I), as well as the related ring system, (II), were found to be substrates for the enzymic reduction,



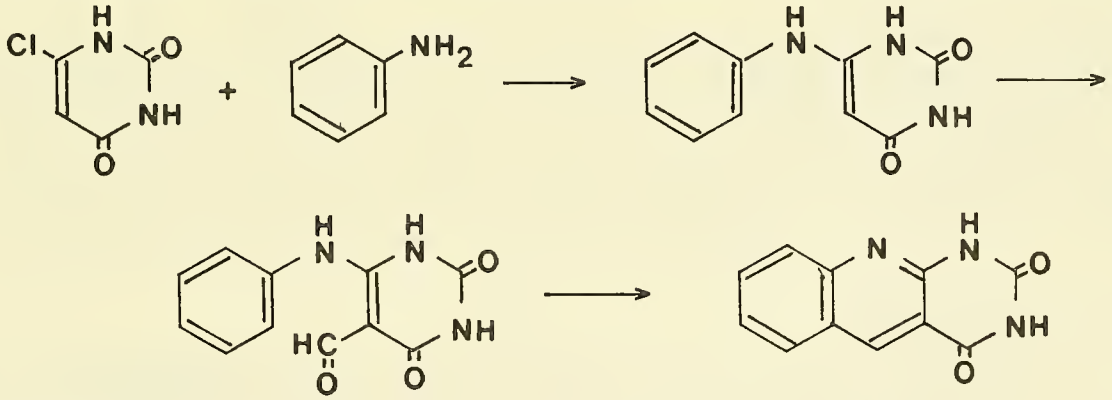
(I)



(II)

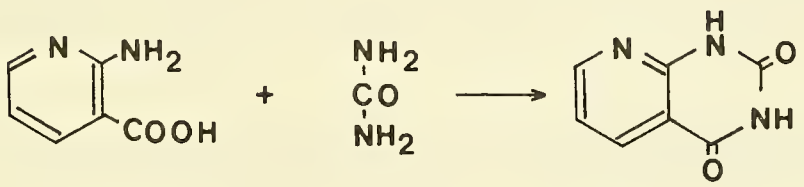
it is of interest to examine the enzymic and chemical reactivities of analogues containing the minimum heterocyclic systems related to the natural cofactor. For this purpose, the following compounds, (III), (IV) and (V), were synthesized.

(a)



(III)

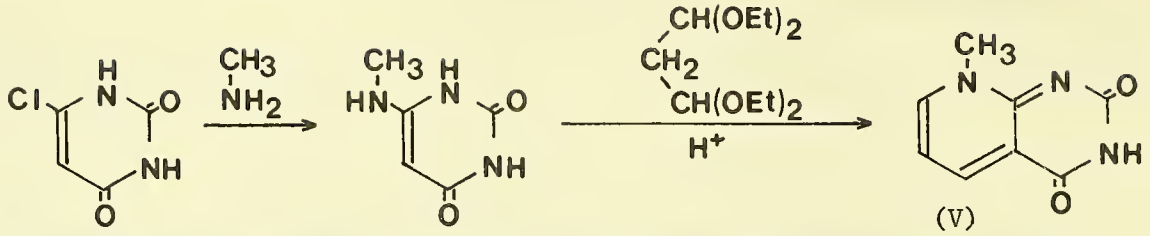
(b)



(IV)

41

(c)

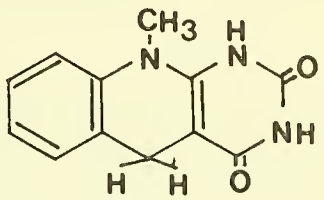


(V)

The structures of these compounds were consistent with their spectroscopic properties. Compound (III) represents the parent heterocycle system of (II), while (IV) and (V) are the lower ring-homologues of (III) and (I) respectively.

Chemical reduction of (III), (IV) and (V) were extremely slow and they were not reduced enzymically. Comparison of the reactivities of (II) and (III) thus indicates that the presence of the 8-hydroxy function lowers the redox potential of the molecule.

(B) The chemistry of the dihydro compound (I') can be considered as that of a disubstituted uracil.



(I')

In order to effect degradation of (I') without disturbing the prochiral center introduced through the enzymic reduction, it is desirable to functionalize the double-bond of the uracil portion of the molecule. Therefore, the chemical reactions known to attack the double bond of uracil were attempted on (I'). These reactions are: (a) acid-catalyzed addition of methanol; (b) addition of bromine; (c) addition of hydrogen bromide; and (d) photo-induced addition of methanol. Unfortunately, thus far none of the reactions were successful with (I').

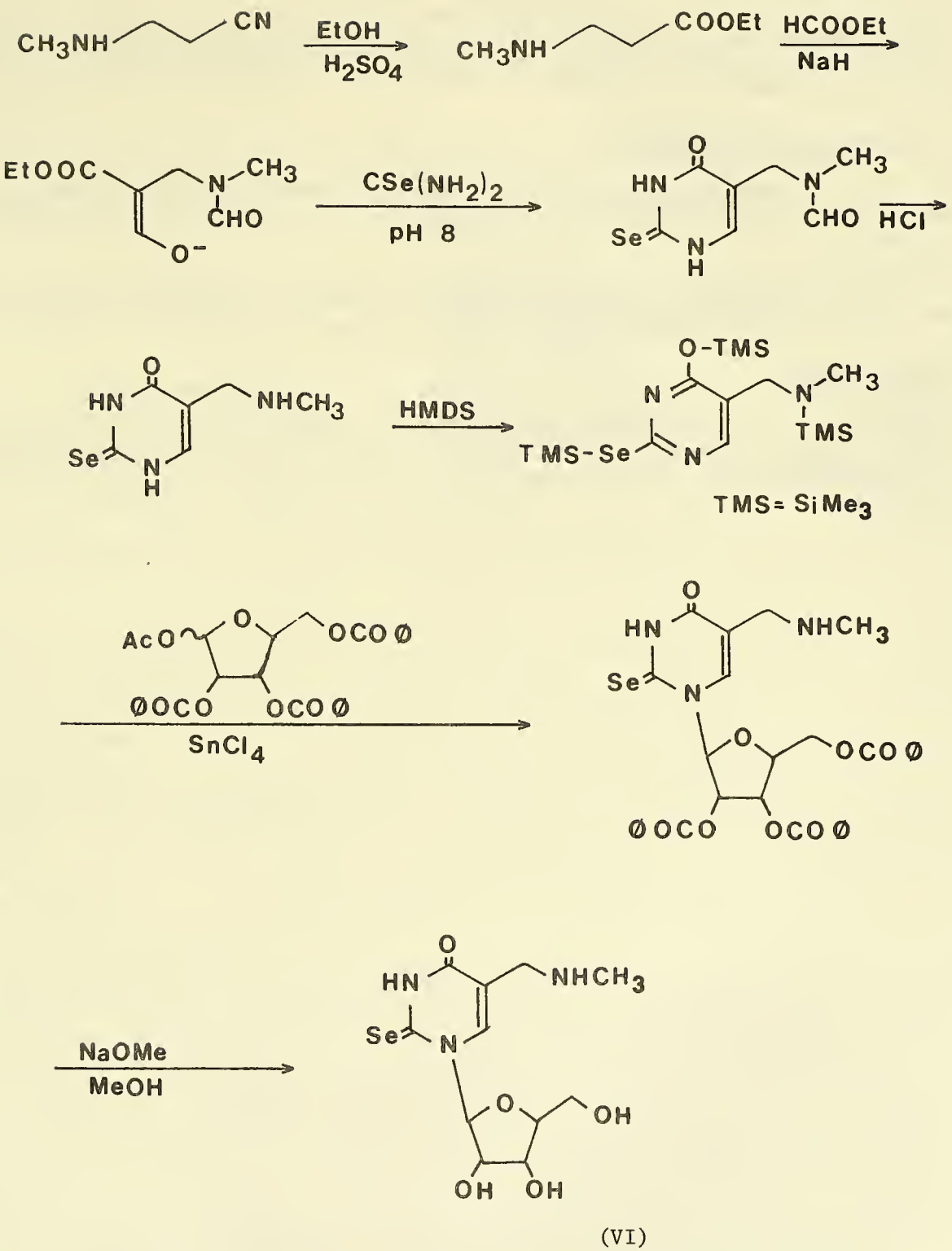
Project Objectives (2)

The presence of a seleno-nucleoside in a tRNA from E. coli was demonstrated by Dr. A. Wittwer. On the basis of the UV spectrum and other properties, the structure, 5-methylaminomethyl-2-seleno-uridine (VI) was proposed for this nucleoside. In order to establish this structure conclusively, it is desirable to have an authentic sample of this novel compound for direct comparison.

Major Findings (2)

Preliminary experiments indicated that a synthesis of 5-methylaminomethyl-2-selenouridine (VI) could be achieved as outlined in Scheme.

42.


CH3NH
CN
EtOH
H2SO4
CH3NH
COOEt
HCOOEt
NaH
EtOOC
N
CH3
CHO
O-
CSe(NH2)2
pH 8
HN
O
Se
N
H
CH3
CHO
HCl
NHCH3
HMDS
O-TMS
TMS-Se
TMS
TMS= SiMe3
AcO
OCOØ
ØOCO
SnCl4
NaOMe
MeOH
OH
OH
OH

(VI)

Scheme

The main difficulty in this synthesis was the coupling of the ribosyl group to the pyrimidine base. So far, this step produced only a very small yield of the desired product (VI), which was characterized by its UV spectrum and elution profile of HPLC.

Proposed Course of Action

(1) To make improvements on the synthesis of the selenonucleoside and to synthesize analogous nucleosides for comparative studies of their chemical properties.

(2) To continue to develop degradative methods of dihydro-5-deazaflavins to be used in stereochemical studies of the enzymic reduction.

Publications

Yamazaki, S., Tsai, L., and Stadtman, T.C.: Analogues of a 8-Hydroxy-5-deazaflavin Cofactor: Relative Activity as Substrates for 8-Hydroxy-5-deazaflavin Dependent $NADP^+$ Reductase from Methanococcus vannielii. Biochemistry 21 934-939, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 00211-09 LB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Mechanism of Cellular Regulation: Regulation of Glutamine Synthetase Inactivation and Degradation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	E. R. Stadtman	Chief, Laboratory of Biochemistry	LB NHLBI
Others:	R. L. Levine	Research Associate (Senior Surgeon, U.S.P.H.S.) Medical Staff Fellow (as of 6/27/82)	LB NHLBI
	P. Z. Smyrniotis	Research Chemist	LB NHLBI
	M. E. Wittenberger	Biological Laboratory Technician	LB NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.4	2.1	1.3

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A. Spectral perturbations associated with the binding of Cibacron blue to glutamine synthetase have been used to monitor protein conformational changes associated with (a) the dissociation of enzyme subunits and (b) the inactivation of glutamine synthetase by mixed function oxidation reactions. B. In the presence of O_2, hypoxanthine and Fe(III), xanthine oxidase catalyzes the inactivation of glutamine synthetase. With low concentrations of xanthine oxidase, the inactivation reaction is markedly stimulated by either ferredoxin, putida redoxin, cytochrome P-450, FMN, or menadione. Roles of superoxide anion, hydrogen peroxide, Fe(III) and hydroxyl radical are indicated by the sensitivity of the inactivation reaction to superoxide dismutase, catalase, EDTA, and free radical scavengers, respectively. The results suggest that glutamine synthetase inactivation involves a Haber-Weiss type mechanism.

PHS-6040
(Rev. 2-81)

Project Description

A. Previous studies in this laboratory have shown that commercial preparations of Cibacron blue dye could be resolved into at least four major components by means of column chromatography. Each fraction elicits a different spectral perturbation when it is bound to glutamine synthetase; moreover, changes in protein conformation associated with the interconversion of glutamine synthetase between relaxed, taut, and subunit dissociated forms can be monitored by spectral changes that accompany binding of the dyes to the enzyme.

In an effort to develop a method that could be used to quantitate the amounts of inactive glutamine synthetase in purified preparations isolated from different bactches of cells, commercial preparations of Cibacron blue dye and purified dye fractions derived there from were tested for their ability to elicit different spectral perturbations when bound to native and inactive forms of glutamine synthetase. Because a mixture of ascorbate, Fe(III) and O_2 mimics enzyme mediated mixed function oxidative inactivation of glutamine synthetase (see last year's report), ascorbate inactivated glutamine synthetase was used for these studies.

B. Previous studies showed that inactivation of glutamine synthetase is catalyzed by a mixed function oxidation system comprised of a flavo protein (putida redoxin reductase) and a nonheme iron electron carrier protein (putida redoxin), and that the further addition of cytochrome $P\text{-}450_c$ to this system increases the rate of glutamine synthetase inactivation and alters the mechanism of oxygen inactivation. Other studies (see last year's report) have shown that similar if not identical mechanisms are involved in the inactivation of glutamine synthetase by various enzyme mediated mixed function oxidation reactions. To gain further insight as to the nature of the activated oxygen species involved, we have examined the ability of nicotinic acid hydroxylase and xanthine oxidase to catalyze inactivation of glutamine synthetase. Both nicotinic acid hydroxylase and xanthine oxidase are flavo proteins that contain nonheme iron sulfide centers and also possess novel molybdenum cofactor derivative at the active-site. In addition, oxidation reactions catalyzed by xanthine oxidase are shown to produce both superoxide anion and H_2O_2.

Major Findings

(1) Commercial preparations of Cibacron blue dye and each of the four major subfractions contained therein produce characteristically different spectral changes when bound to native as compared to ascorbate inactivation glutamine synthetase. With dye fraction I, the difference spectrum between dye + native and dye + inactive enzyme undergoes complex time-dependent changes that are associated with irreversible binding of the dye to the enzyme. In contrast, with dye fraction II, the difference spectrum observed between native, and either 50% inactivated or fully inactivated enzyme, shows a simple maximum at about 660 nm, the amplitude of which is proportional to the amount of inactive enzyme. This dye fraction can therefore be used to quantitate the amount of inactive enzyme in different enzyme preparations.

In earlier studies, it was noted that the spectral perturbations of the dye bound to dissociated subunits was considerably greater than that obtained with native enzyme. By means of ultrafiltration techniques or high speed sedimentation analysis, it was demonstrated that only one molecule of dye is bound to each subunit of glutamine synthetase in both the native and dissociated enzyme configur-

ation. Therefore the greater spectral perturbation obtained with the dissociated subunits reflects a difference in the protein configuration rather than in the number of dye molecules bound per subunit. These studies illustrated further the fact that Cibacron blue dye can be used to monitor conformational changes in protein structure.

(2) In the presence of their electron donors (hypoxanthine or NADPH, respectively), high concentrations of either xanthine oxidase or nicotinic acid hydroxylase are able to catalyze rapid inactivation of glutamine synthetase in an O_2 and Fe(III)-dependent reaction. This inactivation is inhibited by catalase, Mn^{2+} and superoxide dismutase, histidine and free radical scavengers (dimethylthiourea, mannitol, or dimethylsulfoxide) indicating that superoxide anion, H_2O_2 and hydroxyl radical are likely involved. With low concentration of either xanthine oxidase or nicotinic acid hydroxylase, the rate of glutamine synthetase is barely detectable but is enhanced enormously by further addition of either a nonheme iron sulfide electron carrier protein, such as ferredoxin or putida redoxin, or one electron carrier such as menadione or cytochrome P-450. The sensitivity of the xanthine oxidase catalyzed inactivation reaction to inhibition by radical scavengers and superoxide dismutase is quite variable depending upon the concentration of Fe(III) and whether or not the system is coupled with ferredoxin, redoxin, menadione or P-450. In general, the results are consistent with the conclusion that inactivation of glutamine synthetase involves the reduction of Fe(III) to Fe(II) and peroxidation of the Fe(II) at or near the catalytic site to generate hydroxyl radical (or another activated oxygen species) which then reacts with a histidine residue (see R. Levine's report) the modification of which leads to inactivation of the enzyme.

Significance to Biomedical Research

It is well established that the enzyme levels are a function of the rates of synthesis on the one hand and the rates of degradation on the other, and that the rates of enzyme degradation of different enzymes vary greatly and are differentially affected by variations in the nutritional state of the cell. However, almost nothing is known about the mechanism by which a given enzyme is "marked" for degradation. Results of the present study and other studies in this laboratory demonstrate that glutamine synthetase and a number of other key enzymes in metabolism are rapidly inactivated by mixed function oxidase catalyzed reactions. It was further demonstrated that inactivation of glutamine synthetase and of phosphoglycerate kinase are inhibited by substrates of these enzymes, and that the inactivation of glutamine synthetase renders it susceptible to proteolytic degradation. These results suggest that the inactivation by mixed function oxidation reactions may represent an important step in the regulation of turnover of key enzymes. Such inactivation reactions may also play an important role in the killing of bacteria by nutriphils (see C. Oliver's report) and in the proteolytic activities of macrophages.

Proposed Course of Research

Detailed studies of the xanthine oxidase catalyzed inactivation of glutamine synthetase and of other enzymes will be continued. A model system consisting of dihydroxy malate, Fe(III), and O_2 mimics in many respects the xanthine oxidase system. Studies on the inactivation of glutamine synthetase by this system will be carried out in an effort to identify more exactly the nature of the activated

oxygen species that is involved in the inactivation step.

Publications

Oliver, C. N., Levine, R. L., and Stadtman, E. R.: Regulation of Glutamine Synthetase Degradation. In Ornston, L. N. (Ed.): Experiences in Biochemical Perception. New York, Academic Press, 1982, pp. 233-248.

Oliver, C. N., Levine, R. L., and Stadtman, E. R.: Regulation of Glutamine Synthetase Degradation. In Holzer, H. (Ed.): Metabolic Interconverstion of Enzymes. Berlin, Heidelberg, New York, Springer-Verlag, 1981, pp. 259-267.

Stadtman, E. R., Chock, P. B., and Rhee, S. G.: Interconvertible enzyme cycles in cellular regulation. Curr. Top. Cell. Regul. 18: 79-94, 1981.

Mura, U., Chock, P. B., and Stadtman, E. R.: Allosteric regulation of the state of adenylylation of glutamine synthetase in permeabilized cell preparations of Escherichia coli. J. Biol. Chem. 256: 13022-13029, 1981.

Mura, U. and Stadtman, E. R.: Glutamine synthetase adenylylation in permeabilized cells of Escherichia coli. J. Biol. Chem. 256: 13014-13021, 1981.

Hohman, R. J. and Stadtman, E. R.: Relationship between epitope density and immunoprecipitation of multivalent antigens by bivalent antibody: Immunoprecipitation of adenylylated glutamine synthetase of anti-AMP antibodies. Arch. Biochem. Biophys., in press.

Oliver, C., Fucci, L., Levine, R. L., Wittenberger, M., and Stadtman, E. R.: Inactivation of Key Metabolic Enzymes by P-450-Linked Mixed Function Oxidation Systems. In Proc. 4th Internat. Conference on Cytochrome P-450: Biochemistry, Biophysics and Environmental Implications, Kuopio, Finland, May 31-June 3, 1982, Elsevier/North-Holland, 1982, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00212-11 LB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Regulation of Ammonia-Assimilatory Enzymes in E. coli K12

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Mary Anne Berberich	Research Chemist	LB NHLBI
Others:	Sue Goo Rhee	Research Chemist	LB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.1	0.2

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Genetic and biochemical studies with enterobacteria reveal that nitrogen control in these organisms is quite complex. Nitrogen control can be defined as the adjustment in enzyme synthesis made by cells in response to the availability of nitrogen in the growth medium. The levels of the NH_3-assimalatory enzymes, as well as some amino acid transport systems and catabolic enzymes are affected by this process. The attention of this laboratory has been directed toward elucidating the elements involved in nitrogen control and determining the degree of their functional specificity for the class of NH_3-assimilatory enzymes represented by glutamine synthetase (GS), glutamate dehydrogenase (GDH), and glutamate synthase (GAT). Studies have recently focused on the possibility that an additional regulatory role in nitrogen control might exist for some of the proteins which participate in the cascade for modulation of the enzyme activity of GS. Specifically, the function of uridylyltransferase (UT) in the scheme for the regulation of synthesis of GS has been pursued. An additional element involved in nitrogen regulation has been defined.

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

1. Selection of mutants of *E. coli* K12.

   (a) manifesting alterations in the amount or regulation of the NH_3-assimilatory enzymes (GS, GAT, GDH).

   (b) affecting the enzymes and proteins involved in the modification cascade for GS activity.

2. Biochemical characterization of genetic effects.

3. Genetic mapping of the mutations involved.

Major Findings

1. Selection of mutants. Mutants devoid of uridylyltransferase activity (UTase$^-$) have been described previously (reference 1). Revertants to glutamine-independent growth arise spontaneously when glnD$^-$ is plated on nutrient agar in the absence of glutamine. These have been shown to be phenotypic suppressors distributing approximately 1:3 between UT$^-$AT$^-$ and UT$^-$"C" types. In addition, a series of tn5-induced revertants of glnD$^-$ were also isolated. This procedure also yielded phenotypic suppressors which, however, distributed approximately 3:1 between UT$^-$AT$^-$ and UT$^-$"C" types. In addition, a glutamine auxotroph isolated by DES mutagenesis which very quickly reverted to gln$^+$ was characterized and shown to behave like an ntrB type.

2. Biochemical characterizations. None of the fourteen glutamine-independent revertants of glnD$^-$ are revertants to UT$^+$. Examination of extracts in a coupled assay* for P_{IID}-dependent deadenylylation of $GS_{\overline{12}}$ showed that all of the independent isolates were still UT$^-$ and were therefore phenotypic suppressors. Moreover, the representatives examined had normal levels of P_{II} which could function as substrate in a uridylylation reaction system. However, the suppressors could be divided into two classes on the basis of ATase activity. One group of four isolates is UT$^-$AT$^-$ while a group of ten isolates is UT$^-$AT$^+$.

Members of the UT$^-$AT$^+$ group display high levels of fully adenylylated GS under all conditions of growth. On the other hand, members of the UT$^-$AT$^-$ group have levels of GS which are significantly lower (< 50%) than wild type under repressing and derepressing growth conditions although the fold increase is about the same.

Both UT$^-$"C" and UT$^-$AT$^-$ showed marked sensitivity to MSO and γ-glutamyl hydrazide as compared to the wild type which was resistant to these compounds on the glucose-ammonia overlay test plates. Used as a measure of glutamine transport activity, these conditions clearly discriminated between the control strains. The growth of glnpl, a well characterized mutant with a three-fold elevation of the

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*It has been determined by Dr. Sue Goo Rhee that both the parent glnD$^-$ of these strains and a tn-10 induced glnD$^-$ obtained from Pahel and Magasanik show low levels of UT activity as measured by incorporation of UMP^{32} into P_{II} protein. The label could be liberated following digestion by snake venom phosphodiesterase. It is conceivable that the suppressors described above augment the function of a damaged UT *in vivo* and therefore represent the modification of elements which normally interact with the enzyme in the cell.

specific glutamine binding protein, was markedly inhibited, whereas that of GH_{20}, a mutant devoid of this protein, was unaffected. These results suggest that, although UT^-AT^- has very low GS under these conditions, the glutamine transport system is elevated in both UT^-AT^- and UT^-"C". It would appear that regulation of GS level has been uncoupled from general nitrogen control in UT^-AT^-. The response of the parent UT^- strain to these compounds could not be tested due to its glutamine growth requirement.

Both UT^-AT^- and UT^-"C" strains have elevated levels of GDH as compared with wild type and the UT^- parent which has less than wild type levels of GDH. Like the wild type and UT^- parent, both UT^-AT^- and UT^-"C" show marked increases in GDH activity following addition of D-glutamate to growing cells. However, D-glutamate does not evoke an increase in GS in the UT^-"C" strains or in a putative ntrB type.

In Salmonella, mutants in ntrA, B or C fail to use arginine as sole nitrogen source (reference 2). In the strains derived from the E. coli W3102 described here, only the wild type uses arginine as sole nitrogen source. In addition, only the wild type and the UT^-"C" strains described above use aspartate as sole carbon source.

3. Genetic mapping.

(A) ATase-mutations. Assignment of locus to this mutation has been hindered by the lack of a selectable phenotype for recombinants. There are indications that selection for kanomycin resistance of $glnD^-$ favors the appearance of the UT^-AT^- phenotypic suppressors although identity between the tn5 insertion element and $ATase^-$ has not been established. Part of the confusion is due to non-specific effects of mutations on sensitivity to kanamycin (tn5 is an insertion element which also carries resistance to kanamycin, an amino glycoside antibiotic). Thorbjornadottir, S. H. et al. (reference 3) have described several types of spontaneous mutations which cause resistance to kanamycin and related aminoglycosides in E. coli. Two types, unc and ecf, apparently affect the rate-limiting step of accumulation by decreasing the electrochemical gradient or by altering the cell membrane. Other types cause ribosomal alterations. The effects described by these investigators are cumulative and operative within the concentration range used to select for kanamycin resistant insertion mutations.

The results of many genetic crosses indicate that the property of kanamycin resistance affects some cellular component with which ATase interacts in the course of its activity. For instance, using Ø prepared aginst the UT^-"C" kanamycin resistant isolate as donor, apparent AT^- types are obtained with $glnD^-$, wild type and glnA recipients when selection is on the basis of KAN^r. Controls without Ø donors are sterile on kanamycin media. The apparent $AT^-(KAN^r)$ could be shown to be ~ 20% linked to glnA and ~ 80% linked to metB. However, no increase in AT activity could be observed in strains carrying the appropriate multicopy plasmid introduced by means of the methionine coverage. Only the latter strains were assayed for ATase in the purified assay system, i.e., extracts of apparent AT^-metB^+ recombinants were not tested for the absence of AT^- activity. The same relationship appears when using a lysogenzying Ø carrying a chloramphenicol-resistance element as the donor of AT^- where chloramphenicol is included in all transduction plates. Again, appropriate controls of recipient without Ø vector were sterile.

The AT^- trait is not closely linked to glnD, glnF, glnB, pyrE, or ilvECDA. A functional linkage to glnA, metB and argH may exist. AT is not carried by ple 41-35 (glnA) or ple's 22-40, 39-34, 17-1 (metB).

(B) Constitutive suppressors of glnD$^-$. Only one of ten UT$^-$"C" isolates is linked to glnA by p1 transduction. This probably belongs to a class of constitutives defined as ntrB (reference 2). Although UT$^-$ has not been removed from the suppressor strains, evidence indicates that the property of constitutivity is not dependent on the presence of UT$^-$. Introduction of plc 38-39 (a multicopy plasmid carrying glnD, reference 1) has no effect on constitutivity although, as might be expected, $\bar{n}$ GS is lowered.

Two strains from the unlinked group of phenotypic suppressors were chosen for further genetic study -- one from the tn5 selection which is KAN^r and one from the group of spontaneous revertants. To date none of the various attempts at genetic recombination have been successful in achieving transfer of the constitutive character from either of these donor strains. However, Hfr transfer to the constitutive strains indicated that an exceptionally high percentage of the gal$^+$ exconjugants were no longer constitutive. Transduction to gal$^+$ with p1 and scoring for loss of the "C" phenotype by colony assay localized the constitutive element at 17'. Since none of the transductants to loss of "C" required glutamine for growth, the possibility that the phenotypic suppressors (and perhaps the UT$^-$ parent) also carry mutations in the glnR region is under consideration. Also being examined is the possibility that "C" represents a special class of AT$^-$. As noted above, some AT$^-$ could be generated by transduction using the KAN^r"C" plcamØ.

Strains were constructed for three point crosses. The linkages observed using plØ from gal$^+$nadA as donor were: nadA-gal 63%; "C"-Nad 78%; "C"-gal 77% which would suggest the tentative order nadA-"C"-gal. However, in this case, scoring for "C" in colonies from LB glutamine plates rather than glucose-ammonia-glutamine or galactose ammonia glutamine plates appeared to increase the numbers of "C" types and consequently decrease the % linkage so that the order would appear "C"-nadA-gal or nadA-gal-"C". With plØ prepared from gal$^+$bisA::tn10, the linkages observed were: gal-C 33%; bis-gal 3%; bis-C 50% which would favor the order nadA-gal-"C"-bisA. Further work to establish the definitive order of these genes is in progress. Their relationship to the glutamine transport genes at this location (reference 4) is also under study.

By means of F'100, which spans the galactose operon, it could be shown that the gene determining constitutivity is trans recessive and therefore must code for a diffusible product. In the presence of the F'100, the UT$^-$"C" strains were no longer constitutive for GS. "Curing" of the F' by the classical cridine orange method resulted in the recovery of the constitutive phenotype, i.e., the gal$^-$ colonies had high levels of GS under repressing conditions. The determination that "C" is carried on F'100 makes an analysis of its product by cloning techniques a real possibility.

Proposed Course of Research

1. Proceed with genetic analysis of ATase$^-$ and "C" type mutations.

2. Continue to explore the hierarchy of nitrogen control.

3. Construct a strain useful for specific removal of glnD$^-$ from phenotypically suppressed strains.

4. Elucidate the mechanism of the D-glutamate effect.

References

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Thorbjornadottir, S. H., Magnusdottir, R. A., Eggerston, G., Kagan, S., and Andresson, O.: Mutations determining generalized resistance to aminoglycoside antibiotics in E. coli. Mol. Gen. Genet. 161: 89-98, 1978.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00217-07 LB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Purine Nucleosides As Biological Effectors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.: Donita L. Garland Senior Staff Fellow LB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.0	0.2

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The 35,000 Dalton protein in brain supernatants that becomes labeled in the presence of nucleosides is glyceraldehyde-3-phosphate dehydrogenase. The labeled intermediate has been identified as the 3-phosphoglyceryl-S derivative of G3PDH. The precursor, glyceraldehyde-3-phosphate, is likely formed as the result of a series of reactions starting with nucleoside phosphorylase and phosphopentose mutase followed by the enzymes of the nonoxidative part of the pentose pathway. Protein kinase activity has been demonstrated in both the cortical and nuclear fractions of bovine lens. Histone II-AS and endogenous proteins can be phosphorylated. In the cortical fraction, a 51,000 band is phosphorylated. In the nuclear fraction, phosphorylated bands have molecular weights of 17,000-18,000, 20,000, 22,000, < 10,000 and about 61,000. The first three are presumed to be subspecies of crystallins. Purified preparations of α-, β-, and γ-crystallins could not be phosphorylated by either the cortical or nuclear kinases or rabbit muscle cAMP-dependent protein kinases (peak I or peak II). The presence of phosphoserine or phosphothreonine could not be demonstrated in any of the crystallin preparations.

PHS-6040
(Rev. 2-81)

Project Description

I. The long term goals of this research are to further understand some of the control processes in purine metabolism, and to understand the interrelationships of the resulting metabolites with other metabolic pathways and cellular processes. The immediate objective was to identify the protein that became labeled with ^{32}P in the presence of nucleosides in cell-free extracts of porcine brain and elucidated the mechanism of labeling.

II. Studies from other laboratories using [^{32}P]NMR demonstrate the presence of several phosphate containing compounds. One significant peak remains unidentified. The immediate goal was to determine if this compound was a phospho-crystallin. This was approached by determining (1) if any of the crystallins could be phosphorylated by endogenous or exogenous protein kinases and (2) if purified crystallins contain phosphate. Fractionated lens samples were prepared by Dr. John Clarke, Massachusetts Institute of Technology.

I. Major Findings

1. The 35,000 Dalton protein in brain supernatants that becomes labeled in the presence of purine nucleosides and [^{32}P]Pi is glyceraldehyde-3-phosphate dehydrogenase.

2. The labeled intermediate has been identified as the 3-phosphoglyceryl-S derivative of G3PDH. It is presumed that ^{32}P-labeled glyceraldehyde-3-phosphate combines with G3PDH, and is oxidized by NAD to form the catalytic thiolester intermediate.

3. Glyceraldehyde-3-phosphate is most likely formed as the result of a series of reactions starting with nucleoside phosphorylase and phosphopentose mutase followed by the enzymes of the nonoxidative part of the pentose pathway. This sequence is supported by two observations. The addition of ribose-1-phosphate inhibits the labeling using [^{32}P]Pi. When [U-^{14}C]adenosine is used, ^{14}C is incorporated with the appropriate stoichiometry. One observation is not consistent with this reaction sequence. The ^{32}P labeling of G3PDH occurs in the presence of 5 mM EDTA. Transketolase, from other sources, has a requirement for divalent cation. Thus, the postulated pathway for the formation of glyceralde-3-phosphate must be confirmed.

4. GPDH is labeled when uridine, but not cytidine, is added. This suggests the presence of a pyrimidine nucleoside phosphorylase or uridine is also a substrate for the purine nucleoside phosphorylase. Deoxyinosine and deoxyguanosine should not be utilized, however, 5-15% of the maximal labeling is observed with these nucleosides. These preparations are sufficiently pure by HPLC analysis that these results cannot be explained by contaminating ribosides. These results demonstrate efficient flow of metabolites through the nonoxidative portion of the pentose pathway. Labeling of G3PDH is observed at even 1 μM inosine. This may represent an important means of energy production in some tissues. ATP is formed at essentially no cost to the cell. The results also demonstrate an interrelationship between nucleotide metabolism and glycolysis.

II. Major Findings

1. The cortical and nuclear fractions of bovine lens have endogenous protein kinase activity. In the presence of cAMP, histone II-AS was phosphorylated with

rates of about 28 pmol/min/mg of cortical protein and 20 pmol/min/mg of nuclear protein.

2. When cortical and nuclear fractions were incubated in the presence of [γ-^{32}P]ATP, endogenous phosphorylation in each fraction was observed. The pattern of labeling in the two fractions was totally different. In the cortical fraction, the majority of the phosphate coelectrophoresed with a band of about 51,000 molecular weight and a small amount was found in a 20,000 band.

In the nuclear fraction, the majority of the phosphate was incorporated into a band corresponding to a molecular weight of 17,000-18,000. Bands which have M_r's of 61,000, 20,000, 23,000 and < 10,000 also contained some ^{32}P. The 17,000-18,000 band is likely a species of γ-crystallin that is found primarily in the nuclear fraction. The bands at 20,000 and 23,000 are likely subspecies of α- and γ-crystallin. The endogenous phosphorylation with the cortical fraction did not require the addition of cAMP, whereas the labeling in the nuclear fraction was dependent on the addition of cAMP. The addition of protein kinase inhibitor (250 µg/ml) had essentially no effect on phosphorylation in cortical fraction, whereas there was about a 20% decrease in the rate of phosphorylation in the nuclear fraction. Phosphorylation with both the cortical and nuclear fractions was optimal at about 0.5-1.0 mM $MgCl_2$ when ATP concentration was 100 µM. The patterns of labeling with ^{32}P were unchanged with varying Mg^{++} concentration. The addition of protein kinase (peak I and Peak II) did not increase the incorporation for either the cortical or nuclear fractions or change the pattern of phosphorylation as analyzed by gel electrophoresis and autoradiography. Preincubation of the lens fractions prior to the addition of ATP did not increase the ^{32}P incorporation. Preincubation of the fractions with alkaline phosphatase attached to Sepharose heads did not increase the phosphorylation. The addition of Ca^{++} or sodium fluoride had no effect on ^{32}P incorporation.

3. Partially purified preparations of α-, β-, and γ-crystallins were obtained from Dr. Sam Zigler (National Eye Institute). They were used as substrates for both the cortical and nuclear endogenous kinases and for the rabbit muscle peak I and peak II cAMP-dependent protein kinases. α- and β-Crystallins were not phosphorylated. A very low level of phosphorylation was observed with the preparation of α-crystallins. These results are not necessarily unexpected since the crystallins are, while a mixture, primarily derived from the cortical fibers. Phosphorylation of bands that correspond to crystallins was only observed in the nuclear fraction.

4. The purified preparations of α-, β- and γ-crystallins were analyzed for phosphate content by amino acid analysis and by the method of Ames after ashing the proteins. There was no phosphate detected by either method for any of the crystallins. Allowing for even a 50% loss during hydrolysis, phosphorylation of less than 0.5% of the molecules would have been detected. At the minimum, these results demonstrate that the crystallins are not highly phosphorylated. These results demonstrate differences between the nuclear and cortical fractions in the endogenous protein kinases and in the proteins phosphorylated. These differences could reflect changes in cyclic nucleotide metabolism, regulation of protein kinase activity, and/or substrates with aging of the lens cells. In addition to gaining insight into the metabolism of this unique tissue, it is of interest whether these changes contribute to the temperature-dependent opacification which occurs in the nuclear region of the lens.

Proposed Course of Research

1. Confirm the proposed pathway for glyceralde-3-phosphate production.
2. Investigate the effect of inosine on G3PDH.
3. Reexamine this pathway in other tissues including spleen and thymus.

Relevance to Medicine

It is now apparent that purine nucleosides serve as biological modulators in a number of tissues. Nucleosides have neurotransmitter function; they effect the synthesis and release of hormones and coronary blood flow. Adenosine modifies the concentration of cAMP in many cell types by regulating the activity of adenylate cyclase. Inosine and guanosine have inotropic and chronotropic effects on the myocardium. Inosine and hypoxanthine may be endogenous ligands for the benzodiazepam binding sites in brain. Deficiencies in the nucleoside metabolizing enzymes, adenosine deaminase and purine nucleoside phosphorylase, which lead to increased levels of adenosine or inosine and guanosine, respectively, are associated with B-cell and T-cell dysfunction. In the case of purine nucleoside phosphorylase deficiencies, the increased inosine and guanosine levels lead to neurological defects. The precise role of the nucleosides and the underlying biochemical mechanisms are not well established in any of these phenomena.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00224-05 LB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Enzyme Mechanism and Regulation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Charles Y. Huang	Research Chemist	LB NHLBI
Other:	Wei-Chao Ni	Visiting Fellow (started 3/30/82)	LB NHLBI

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Laboratory of Biochemistry

SECTION
Section on Metabolic Regulation

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.8	1.5	0.3

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

(1) The dissociation constant for the complex between cyclic nucleotide phosphodiesterase and fully-liganded calmodulin at pH 7.0, 25°C, has been redetermined from six sets of data to be $(1.2 \pm 0.2) \times 10^{-11}$ M. (2) The mechanism of activation of proteinase B in yeast at pH 5 involves the loss of inhibitory effect of proteinase B inhibitor, I_B, due to a slow irreversible conformational rearrangement. Isoelectrofocusing experiments revealed that I_B can exist in four forms which differ in susceptibility to loss of inhibitory effect by pH 5 treatment. (3) The theory for the continuous variation method for the determination of binding stoichiometry has been extended to proteins exhibiting cooperative binding effects. The theory is applicable to both the sequential model of Adair and the concerted model of Monod.

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Project Description

Objectives: (1) To gain knowledge of the regulatory and catalytic mechanisms of enzymes. (2) To develop or improve methods and theories applicable to the study of enzymes.

Major Findings

1. The dissociation constant for the cyclic nucleotide phosphodiesterase-calmodulin complex at saturating level of Ca^{2+}, K_a, has previously been determined to be on the order of 10^{-10} M at pH 7.0, 25°C. This value carries some uncertainties because it is obtained from a single determination according to the following relationship:

$$K_a = CM_{0.5} - E_o/2$$

where $CM_{0.5}$ is the total calmodulin concentration at which half maximal activation of phosphodiesterase is observed, and E_o is the total concentration of phosphodiesterase. The K_a value has been redetermined from a series of experiments where the activation of phosphodiesterase by a given calmodulin concentration was measured as a function of free Ca^{2+} concentration. The free Ca^{2+} concentrations at half maximal activation of phosphodiesterase were used to calculate K_a according to the following equation:

$$\frac{\dfrac{CM_o \cdot C^4}{K_a K_1 K_2 K_3 K_4}}{\dfrac{CM_o \cdot C^4}{K_a K_1 K_2 K_3 K_4} + \phi} = 1/2$$

where $\phi = 1 + C/K_1 + C^2/K_1K_2 + C^3/K_1K_2K_3 + C^4/K_1K_2K_3K_4$, C = free Ca^{2+}, and K_1, K_2, K_3, K_4 = dissociation constants for the four Ca^{2+} from calmodulin.

The K_a's calculated from data obtained at total calmodulin concentrations of 20.9 nM, 58.8 nM, 0..108 µM, 0.216 µM, 1.93 µM, and 11.6 µM all fall within the range of $(1.2 \pm 0.2) \times 10^{-11}$ M, indicating that the affinity of phosphodiesterase for the fully-liganded calmodulin is almost one order of magnitude higher than previously determined. The new value is more reliable since it is estimated from six sets of data and the equation used does not contain the enzyme concentration term. Uncertainty of the concentration of active phosphodiesterase is a major source of error in previous K_a calculations.

2. The slow activation of proteinase B in yeast at pH 5 was thought to be due to the degradation of proteinase B inhibitor, I_B, by proteinase A. However, it was found that, when pepstatin was added to completely inhibit proteinase A activity, the activation of proteinase B was not hampered. We found that I_B loses its inhibitory effect irreversibly on incubation at pH 5.

The I_B used initially was prepared by boiling the post-DEAE-cellulose proteinase B-I_B mixture for 10 minutes to remove the heat labile proteinase B. The I_B so prepared was incubated at pH 5 or pH 7, 30°C, and its ability to inhibit proteinase B was tested at various time intervals. At pH 5, I_B gradually lost its inhibitory effect and was totally ineffective after 18 hours. In contrast, at pH 7, the

inhibitory effect of I_B remained unaltered for 18 hours.

When I_B was prepared by boiling the yeast cells, however, the inhibitory effect could not be completely destroyed by incubation at pH 5. Further purifications of the I_B by gel filtration followed by isoelectrofocusing revealed the presence of four I_B's having P_I's at 4.65, 6.2, 6.8, and 7.5. The 4.65 fraction could not be destroyed by incubation at pH 5, the 7.5 fraction was partially destroyed (20-30%); and the 6.2 and 6.8 fractions were essentially destroyed. The different susceptibilities to pH 5 incubation of these I_B's explain why the stability of I_B at acid pH's reported by different laboratories varied significantly.

The loss of inhibitory effect appears to be the result of a slow, irreversible conformational rearrangement. It was not due to the presence of trace amounts of proteinase A, proteinase B, carboxypeptidase, or aminopeptidase. None of these activities was found in various assays. Besides, if there is an unknown proteinase that survived the boiling, then the pH 5-treated I_B may still contain this proteinase. In this case, addition of the pH 5-treated protein solution to the untreated preparations should accelerate the degradation of I_B at pH 5. It was found that addition of pH 5-treated I_B did not cause any increase in the rate of loss of untreated I_B at pH 5. Also, when the inactive I_B was incubated at pH 7 for up to 24 hours and assayed at different time intervals, no recovery of inhibitory effect could be detected. The result is consistent with the notion that the conformational change is irreversible (in collaboration with Professor G. Magni, University of Camerino, Camerino, Italy).

3. The continuous variation method for the determination of binding stoichiometry has been extended to proteins exhibiting cooperative binding effects.

For the Adair model, let us use the simplest case of a dimeric enzyme as an example:

$$\frac{\Sigma}{E_o} = \frac{2\,(L/K_1 + L^2/K_1K_2}{1 + 2\,L/K_1 + L^2/K_1K_2} \qquad (1)$$

where Σ = summation of all enzyme-ligand complexes, L = free ligand concentration, and K_1 and K_2 and the two dissociation constants.

With the continuous variation method, the total protein and ligand molar concentration is held at a constant level C_o such that

$$E_o + L_o = C_o$$

or $$X + Y = 1 \quad (X = E_o/C_o,\ Y = L_o/C_o)$$

Thus, substitution of the relationship

$$L = L_o - \Sigma = C_o - C_o\,X - \Sigma$$

into Equation 1 and differentiating it with respect to X leads to the following expression:

$$\left(\frac{d\Sigma}{dX}\right)_{X \to O} = \frac{2\,C_o^2\,(K_2 + C_o)}{K_1K_2 + 2\,K_2\,C_o + C_o^2} \qquad (2)$$

$$\left.\frac{d\Sigma}{dY}\right)_{Y\to O} = \frac{2\,C_o^{\,2}}{K_1 + 2\,C_o} \qquad (3)$$

at the intersection point of the lines with these limiting slopes, we have

$$\frac{Y_i}{X_i} = \frac{K_1K_2 + (K_1 + 2\,K_2)\,C_o + 2\,C_o^{\,2}}{K_1K_2 + 2\,K_2\,C_o + C_o^{\,2}}$$

$$= 2 \text{ when } C_o \gg K_1,\ K_2$$

Thus, the stoichiometry can be determined by the mole fractions of E and L at the intersection point, X_i and Y_i. The mathematical treatment is valid for any Adair type of enzymes with n binding sites. Similar treatment can be applied to the Monod model to demonstrate that theoretical foundation exists for using the continuous variation method to determine the binding stoichiometry for the Monod type of enzymes provided the experimental condition that C_o is much greater than all the ligand dissociation constants is fulfilled.

Proposed Course of Research

1. The mechanism of action and the identification of phosphoprotein substrates of "calmodulin binding protein" as a phosphoprotein phosphatase.

2. The Ca^{2+}-dependent activation of glycogen phosphorylase kinase with respect to calmodulin as the δ subunit and as the exogenous regulator.

3. Regulatory mechanism of protein phosphatase F_C by the activators F_A, Me^{2+}, and ATP.

4. Regulation and activation of yeast proteinases.

Publications

Huang, C. Y., Rhee, S. G., and Chock, P. B.: Subunit cooperation and enzymatic catalysis. Ann. Rev. Biochem. 51: 935-971, 1982.

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Chau, V., Huang, C. Y., Chock, P. B., Wang, J. H., and Sharma, R. K.: Kinetic Studies of the Activation of Cyclic Nucleotide Phosphodiesterase by Ca^{2+} and Calmodulin. In Hidaka, H. (Ed.): Calmodulin and Receptors, in press, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00225-05 LB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Inactivation of Glutamine Synthetase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.: Rodney L. Levine (Research Associate, Senior Surgeon, USPHS) (Medical Staff Fellow as of 6/27/82) LB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.7	0.3

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In bacteria, glutamine synthetase is "marked" for subsequent proteolytic degradation by a covalent modification. This modification involves oxidation of a specific histidine residue. A cyanogen bromide fragment containing the altered histidine has been isolated. The peptide has only a few residues (<10) and is very hydrophilic in behavior.

Glutamine synthetase which has been oxidatively modified in vitro reacts with 2,4 dinitrophenylhydrazine to form a hydrazone. The molar absorptivity is proportional to the extent of oxidative modification. When purified preparations of glutamine synthetase were isolated by our usual method, they also formed phenylhydrazones. The extent of reaction was inversely proportional to their specific activity. Thus, the oxidative modification probably occurs in vivo and accounts for variability in specific activity of purified enzyme preparations.

Oxidative modification may provide another mechanism for regulation of metabolic activity by covalent modification of proteins. In particular, the modification appears to mark glutamine synthetase for proteolytic degradation.

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Project Description

Objectives: Previous reports described an oxidative modification of glutamine synthetase which appears to "mark" the protein for subsequent proteolytic degradation. The objectives of this project are determination of the chemical nature of the oxidative modification, purification and characterization of the inactivating system and the proteolytic system from cells; and assessment of the physiologic controls which regulate the proteolysis of specific proteins.

Major Findings

1. Confirmation that the modification involves a single histidine residue. Using fluorometric detection with an amino acid analyzer, we found that the oxidatively modified glutamine synthetase had lost only one of 16 histidine residues. No other changes were detected. In collaboration with Dr. J. Farber, E. coli were grown on H-3 or C-14 histidine, and the labelled glutamine synthetase was isolated. Study of the oxidatively modified enzyme confirmed loss of one histidine residue. Although the products were not identified, at least five compounds were formed following acid hydrolysis of the modified protein.

2. Other enzymes are susceptible to oxidative modification. Various enzymic and non-enzymic systems oxidatively modify glutamine synthetase. Working with a diaphorase-dependent system, Drs. Fucci and Stadtman demonstrated oxidative modification (with loss of enzymic activity) of several other enzymes. One of these, phosphoglycerate kinase, was subjected to amino acid analysis. It too had lost a single histidine residue. (Modifications in other amino acids might also be present.) Thus, at least one other enzyme undergoes an oxidative inactivation with loss of a single histidine, while the other histidine residues remain intact.

Three enzymes thought to contain a histidine residue at the active site were tested with the ascorbate/oxygen/iron system described previously. Each was inactivated: creatine kinase, pyruvate kinase, and rat liver glutamine synthetase. Since oxidative modification of bacterial glutamine synthetase can be regulated by substrates and products, pyruvate kinase was studied for such effects. The high energy phosphate compounds ATP or phosphoenol pyruvate protected the enzyme from inactivation, while ADP or pyruvate had no effect. Not all enzymes are oxidized by this system. Lysozyme contains a single histidine residue, although it is not thought essential for catalytic activity. When subjected to the ascorbate system, lysozyme activity was not affected. Moreover, amino acid analysis confirmed that the histidine was not altered.

3. Isolation of a peptide containing the altered histidine. A key objective of this project is the characterization of the altered amino acid which results from oxidative modification. Again in collaboration with Dr. J. Farber, the cyanogen bromide peptide containing the altered histidine has been isolated. The peptide has not yet been analyzed in detail. However, it is small (<10 residues) and is distinctly hydrophilic on reverse-phase chromatography. The peptide contains two histidine residues, only one of which is oxidatively modified.

4. The oxidative modification occurs *in vivo*. In collaboration with Dr. S. Shaltiel, the ascorbate-modified glutamine synthetase was found to react with carbonyl reagents (phenylhydrazine, 2,4 dinitrophenylhydrazine, and *ortho*-phenylenediamine). Partially-inactivated preparations of enzyme were prepared using the ascorbate system. The molar absorptivity of the 2,4 dinitrophenyhydrazone was found to be proportional to the extent of inactivation (and to the loss of the histidine residue).

Using our standard purification procedure, preparations of glutamine synthetase usually have a specific activity of 100-120 units/mg. Some preparations occasionally have a lower activity (80-90 units/mg) and some have been as high as 140 units/mg. When 10 randomly selected preparations were tested with 2,4 dinitrophenylhydrazine, all formed a phenylhydrazone. Their molar absorptivity was inversely proportional to their specific activity. Moreover, the molar absorptivity was predicted from that of the ascorbate-inactivated enzymes. Since the oxidative modification would not likely occur during our purification procedure, we concluded that it occurs *in vivo* and accounts for the variability in specific activity of purified enzyme.

Significance to Biomedical Research

Studies in this and other laboratories provide a rapidly growing list of proteins which are susceptible to oxidative modification. Such a covalent modification could be a general phenomenon, important in cellular regulation as are other covalent modifications. We implicated the modification as a "marker" for proteolytic degradation, but this may not be its only role. Akin to other covalent modifications, a reversing, reducing modification may also occur.

Proposed Course of Research

Characterization of the chemical nature of the modified histidine remains a primary objective of the project. With the peptide in hand, this should be facilitated. With J. Roseman, the proteolytic activity which attacks the modified enzyme will be further purified. The isolated peptide may also facilitate that work, through construction of an affinity purification matrix.

Publications

Levine RL and Federici MM: "Quantitation of aromatic residues in proteins: Model compounds for second derivative spectroscopy." Biochemistry 21: 2600-2606, 1982.

Oliver CN, Levine, RL, and Stadtman, ER: "Regulation of glutamine synthetase degradation." In Ornston LN, editor: Experiences in Biochemical Perception. Academic Press, New York, 1982, p 233-249.

Levine RL: "A rapid benchtop method of alkaline hydrolysis of proteins." Journal of Chromatography 236: 499-502 1982.

Oliver CN, Levine, RL, and Stadtman, ER: "Regulation of glutamine synthetase degradation." In Holzer H, editor: Metabolic Interconversion of Enzymes 1980. Springer-Verlag, Berlin, 1981, p 259-268.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00231-04 LB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Development of a Pure In vitro Interconvertible Enzyme Cascade System

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Emily Shacter-Noiman	Staff Fellow	LB NHLBI
Others:	Earl R. Stadtman	Chief, Laboratory of Biochemistry	LB NHLBI
	P. Boon Chock	Chief, Section on Metabolic Regulation	LB NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Metabolic Regulation

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
A model, in vitro phosphorylation/dephosphorylation cascade system was developed in which the two converter enzymes were bovine cardiac cAMP-dependent protein kinase (type II) and phosphoprotein phosphatase (M_r = 38,000). Both enzymes were purified to near homogeneity. The effects of divalent cations, ATP, NaF, and phosphatase inhibitor-2 on the phosphatase were examined. The kinetic parameters for both converter enzymes were determined using the nanopeptide, leu-arg-arg-ala-ser-val-ala-gln-leu as the substrate, cAMP as an activator for the kinase, and Pi as an inhibitor for the phosphatase. The in vitro cascade was studied at different enzyme substrate and effector levels. In the presence of a constant ATP concentration, a steady-state level of phosphorylation of the nanopeptide is attained. The extent of this fractional modification is modulated by the relative concentrations of the kinase, phosphatase, and effectors. The model cascade exhibits both signal amplification and an increase in sensitivity to variations in effector concentrations. In addition, the results demonstrate that when the concentration of the enzyme-substrate complex is not neglibible, as is often found in vivo, the cyclic cascade is potentially more sensitive to variations in effector concentration than predicted previously using simplified equations.

Project Description

Objectives: To develop a model phosphorylation/dephosphorylation cascade system which can be used to test the theories and equations which describe the cyclic modification of a protein. This is useful both as a tool to study the mechanisms whereby enzymes regulate each other, and as a relatively simple model to aid in our conceptualization of metabolic regulation through cyclic covalent modification.

Major Findings

The model cascade verified and extended the predictions resulting from a theoretical analysis of cyclic cascade systems. It exhibited signal amplification and an increase in sensitivity to variations in the concentrations of single and multiple effectors. The simplified equation previously employed to analyze monocyclic cascades was rederived to account for the tight binding between the kinase and the nanopeptide (K_m = 0.26 μM). The complex equation does predict the positive-cooperative type response observed in this system.

Meanwhile, it was found that bovine cardiac phosphoprotein phosphatase is a predominantly type-2 phosphatase; it is not inhibited by heat stable inhibitor-2, but is inhibited potently by ATP, NaF and PPi/Mg^{2+} either reduced or prevented this inhibition depending upon whether or not Mn^{2+} was present in the assay. The results were interpreted by a model which assumes that ATP, NaF, Mg^{2+} and Mn^{2+} each act by binding to separate sites on the enzyme.

Finally, detailed analysis of the activation of the protein kinase by cAMP disclosed that: (i) complete activation may be attained by binding of only two cAMP molecules to the holoenzyme, and (ii) the activation exhibits positive cooperativity.

Proposed Course of Research

A type-1 phosphatase will be purified so that a bicyclic cascade can be developed. This sytem will employ heat stable inhibitor-1 which, when phosphorylated by cAMP-dependent protein kinase, can inhibit type-1 phosphatase activity. This system will be studied in the same manner as the model monocyclic cascade.

Publications

Wright, D.E., Noiman, E.S., Chock, P.B., and Chau, V.: Fluorometric assay for adenosine 3',5'-cyclic monophosphate-dependent protein kinase and phosphoprotein phosphatase activities. Proc. Natl. Acad. Sci. U.S.A. 78: 6048-6050, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00235-04-LB

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Properties of 8-hydroxy-5-deazaflavin-dependent enzymes from Methanococcus vannielii.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Shigeko Yamazaki	Staff Fellow	LB NHLBI
Others:	Thressa C. Stadtman	Chief, Section on Intermediary Metabolism and Bioenergetics	LB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Section on Intermediary Metabolism and Bioenergetics

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1	0.3

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A Se-containing hydrogenase was purified to near homogeneity from extracts of Methanococcus vannielii. The molecular weight of the enzyme was estimated as 340,000. The enzyme tends to aggregate and occurs also as a larger protein species. Both molecular species reduce the 8-hydroxy-5-deazaflavin cofactor with molecular hydrogen. Se is present exclusively in Mr = 42,000 subunit. A value of 3.8 g atoms of Se/mol of enzyme (Mr = 340,000) was determined. The chemical form of selenium in the enzyme was shown to be selenocysteine. This is the first hydrogenase that has been shown to be a selenoenzyme.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: The 8-hydroxy-5-deazaflavin cofactor (8-OH-5dF1) that is abundant in methane-producing bacteria serves as electron carrier in coupled enzyme systems, such as a formate-$NADP^+$ oxidoreductase system and a hydrogen-NADP+ oxidoreductase system. In Methanococcus vannielii, 8-OH-5dF1, which is reduced by formate (via formate dehydrogenase) or molecular hydrogen (via hydrogenase), serves as cofactor for 8-hydroxy-5-deazaflavin-dependent $NADP^+$ reductase (5-deazaflavin-$NADP^+$ reductase) that reduces $NADP^+$ to NADPH.

The aim of this project is to study the properties of 8-OH-5dF1-dependent enzymes and the mechanisms of the reactions catalyzed by the 8-OH-5dF1-dependent enzymes.

Major Findings

A Selenium-Containing Hydrogenase. Growth of M. vannielii in a formate/mineral salts medium is markedly enhanced by supplements of sodium selenite and tungstate. This stimulatory effect was correlated with the appearance of a selenium-containing formate dehydrogenase complex in the cell. (Jones, J.B. and Stadtman, T.C. (1981) J. Biol. Chem. 256, 656-663).

Three different fractions containing ^{75}Se were separated from extracts of ^{75}Se-labeled M. vannielii by chromatography on a Phenyl-sepharose column under strictly anaerobic conditions. The first radioactive peak fraction contained ^{75}Se-labeled tRNAs and the second radioactive fraction exhibited formate dehydrogenase activity. The third radioactive peak fraction which was free of formate dehydrogenase activity was coincident with hydrogenase activity. To investigate the possibility that the ^{75}Se was an actual component of the hydrogenase, purification of the enzyme was undertaken.

A ^{75}Se-labeled hydrogenase was purified to near homogeneity from extracts of M. vannielii cells grown in the presence of [^{75}Se]selenite. The molecular weight of the enzyme was estimated as 340,000 by gel filtration. The enzyme tends to aggregate and occurs also as a larger protein species ($M_r = 1.3 \times 10^6$). The same phenomenon was observed on native gel electrophoretic analysis. Hydrogenase activity exhibited by these two protein bands was proportional to protein and ^{75}Se content. Both molecular species reduce the natural cofactor, 8-hydroxy-5-deazaflavin, and tetrazolium dyes with molecular hydrogen. SDS gel electrophoresis of ^{75}Se-labeled enzyme showed that ^{75}Se is present exclusively in a 42,000 M_r subunit. A value of 3.8 gram atoms of selenium per mole of enzyme (340,000 M_r) was determined by atomic absorption analysis. The chemical form of selenium in the enzyme was shown to be selenocysteine. This was identified as the [^{75}Se]carboxymethyl and [^{75}Se]carboxyethyl derivatives in acid hydrolysates of alkylated ^{75}Se-labeled protein. The hydrogenase is extremely oxygen sensitive but can be reactivated by incubation with molecular hydrogen and dithiothreitol. This is the first hydrogenase that has been shown to be a selenoenzyme.

Proposed Course of Action

(1) Characterization of Se-containing hydrogenase from M. vannielii will be continued.

(2) The possibility that hydrogenases from other Se-dependent bacteria may be selenoenzymes will be investigated.

(3) The absolute configuration at C-5 of enzymatically reduced 8-hydroxy-5-deazaflavin cofactor will be determined by collaboration with Prof. Tetsuo Shiba, Osaka University, Japan.

Publications

Yamazaki, S., Tsai, L., Stadtman, T.C.: Analogues of 8-hydroxy-5-deazaflavin cofactor: Relative activity as substrates for 8-hydroxy-5-deazaflavin-dependent $NADP^+$ reductase from Methanococcus vannielii, Biochemistry, 21, 934-939, 1982.

Yamazaki, S.: Analogues of 8-hydroxy-5-deazaflavin cofactor: Relative activity as substrates for 8-hydroxy-5-deazaflavin-dependent $NADP^+$ reductase, in Flavins and Flavoproteins (V. Massey and C.H. Williams, Jr., eds)) Elsevier North Holland, New York, 442-446, 1982.

Yamazaki, S.: A selenium-containing hydrogenase from Methanococcus vannielii: Identification of the selenium moiety as a selenocysteine residue, J. Biol. Chem. in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00237-03 LB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Toxicity and Transport of Bilirubin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.: Rodney L. Levine (Research Associate Senior Surgeon, USPHS) (Medical Staff Fellow as of 6/27/82) LB NHLBI

COOPERATING UNITS (if any)

Laboratory of Neurosciences, National Institute on Aging

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Hyperbilirubinemia is probably the most frequently diagnosed and treated condition in the human newborn. Treatment attempts to prevent the entry of bilirubin into the brain, because bilirubin in neurotoxic. The mode by which bilirubin enters the neonatal brain is unknown. We demonstrated that albumin-bound bilirubin enters the brain of experimental animals, following opening of the blood-brain barrier. Since bilirubin entered only the treated hemisphere, the contralateral hemisphere serves as a control within the same animal. Then, utilizing direct carotid arterial infusion of bilirubin, we increased the content of brain bilirubin by about ten-fold. This will permit studies of the effects of bilirubin on cerebral metabolism and may provide a model of neonatal kernicterus.

PHS-6040
(Rev. 2-81)

Project Description

Introduction

Bilirubin is an end-product of heme metabolism in mammals. In vitro, bilirubin appears quite toxic to cells and to subcellular organelles such as mitochondria. The biochemical basis of the toxicity is unknown. Yet, hyperbilirubinemia is the most commonly diagnosed and treated medical problem of newborn infants. The purpose of treatment is to avoid transport of bilirubin into the brain, with its subsequent neurologic sequelae and the syndrome of kernicterus.

Major Findings

We continued our studies of the effect of opening of the blood-brain barrier unilaterally in rats. We demonstrated that we could produce staining of the brain in the treated hemisphere, with the untreated hemisphere providing a control for metabolic and other studies. This unilateral kernicterus should prove particularly useful in identifying the metabolic target leading to bilirubin toxicity.

We developed a rapid method for quantitation of the transport of bilirubin into the brain. The method utilizes extraction by an azeotropic mixture of chloroform/methanol/water followed by spectrophotometric determination of bilirubin by first derivative spectroscopy. This technique eliminated interference by hemoglobin, which had been a major difficulty with other methods.

With the quantitative method available, we then turned to systematic manipulation of our animal model in order to increase the transport of bilirubin into the brain. Additionally, we attempted to minimize cross-over transport into the control hemisphere. These studies are nearing completion, and should permit transport of large doses of bilirubin into the brain.

Significance to Biomedical Research

Current treatment of neonatal hyperbilirubinemia is based primarily on the "free-bilirubin" theory, an unproven concept. This may not be the mechanism of transport and toxicity. The availability of an animal model of kernicterus permits studies on the metabolic and neurophysiologic effects of bilirubin, and it is now utilized in several other laboratories as well as ours.

Proposed Course of Research

1. Complete the systematic examination of experimental parameters in order to increase transport of bilirubin into the brain of the experimental animals.

2. Study the metabolic effects of bilirubin on cerebral metabolism. Specifically, we plan to use the 2-deoxyglucose technique to permit in vivo studies.

Publications

Levine RL, Fredericks WR, and Rapoport SI: Entry of bilirubin into the brain due to opening of the blood-brain barrier. Pediatrics 69:255-259 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00239-03 LB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Regulation and Mechanism of Glutamine Synthetase in E. coli

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Sue Goo Rhee	Research Chemist	LB NHLBI
	Hong Keun Chung	Visiting Fellow	LB NHLBI
Others:	P. Boon Chock	Chief, Section on Metabolic Regulation	LB NHLBI
	Earl R. Stadtman	Chief, Laboratory of Biochemistry	LB NHLBI

COOPERATING UNITS (if any)
Dr. Greg Pahel, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA; Dr. Sae W. Suh, Laboratory of Molecular Biology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Metabolic Regulation

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.3	2.0	0.3

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

(1) A multicopy plasmid vector carrying the glnD gene (structural gene for uridylyltransferase-uridylyl-removing enzyme) was constructed by cloning the EcoR1 generated fragment of the plasmid JA200/PLC 38-39. This strain overproduced UT·UR by 25-30-fold. (2) A large quantity of P_{II} protein was purified from an overproducing strain using simplified procedures. X-ray crystallographic and UV spectral studies on P_{II} were pursued. A tryptic undecapeptide containing covalently bound UMP was isolated and the amino acid sequence of this peptide was established. In addition, the properties of phosphotyrosyl P_{II} generated by treating $P_{II}(UMP)_4$ with micrococcal nuclease were studied. (3) A continuous fluorometric assay for deadenylylation reaction was developed. (4). Monoclonal antibodies specific to various antigenic determinants of E. coli glutamine synthetase were prepared. The hybridoma clones were isolated by the fusion of myeloma cells and spleen cells derived from mice immunized with glutamine synthetase or AMP attached to bovine serum albumin.

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PHS-6040
(Rev. 2-81)

Project Description

Objectives:

(1) To construct a strain overproducing UT-UR enzyme.

(2) To understand the protein structure and function of regulatory protein P_{II}.

(3) To prepare monoclonal antibodies specific to glutamine synthetase and characterize them.

Major Findings

(1) Cloning of the glnD gene in PBR 322. In our previous work, we screened the Clarke-Carbon collection of 2,000 *E. coli* strains (each of which carries a distinct hybrid ColE1 plasmid containing random fragments from the *E. coli* chromosome) and obtained two strains JA/PLC 6-32, JA/PLC 38-39 that carry hybrid plasmid containing fragment encoding the glnD gene (structural gene for UT·UR enzyme). The strains overproduced UT·UR by 14- to 25-fold. Since the level of overproduction of UT·UR in JA200/PLC 38-39 and JA200/PLC 6-32 was not sufficient to facilitate our purification studies, the fusion of the glnD gene and a highly efficient *E. coli* promoter, such as the promoter of the lac operon, was undertaken.

In the first step, the plasmid from JA200/PLC 38-39 was treated with EcoR1 restriction enzyme to generate two fragments. One fragment (12 Kb) was ligated to the EcoR1 site of PBR 322 to generate a recombinant plasmid pglnD1. This strain overproduced UT·UR by 25-30-fold. The size of the structural gene for UT·UR, whose molecular weight is 95,000, is expected to be in the range of 2.6 Kb, which is considerably smaller than the 12 Kb EcoR1 fragment. Therefore, pglnD1 was treated with various restriction enzymes to obtain a smaller DNA fragment containing the glnD gene; Aval produced 7.0, 3.8, 2.8, and 2.5 Kb fragments, PVUI 5.6, 5.0, 2.7, 2.4, and 2.3 Kb fragments, PVUII 5.8, 3.5, 2.8, 2.4, and 1.6 Kb fragments, PST1 7.6, 5.3, 2.7, and 0.4 Kb fragments, and Sal 1 9.8 and 6.6 Kb fragments.

Shotgunning experiments designed to select a recombinant strain carrying the glnD gene were unsuccessful mainly because of the leakiness of glnD strain used in the transformation.

(2) A strain bearing the multicopy plasmid vector carrying the glnB gene (structural gene for P_{II}) was constructed by cloning PVUI generated fragment of the recombinant plasmid PGS1 (with Dr. Greg Pahel). This new strain overproduced P_{II} by 70-fold. The use of this P_{II} overproducing strain allowed us to prepare larger quantities of P_{II}. Table I summarizes a typical purification procedure.

Table I. Purification Summary

Step	Total Protein	Yield	Specific Activity
1. Streptomycin	77g	100%	1
2. DEAE cellulose chromatography	2.8	47	13
3. Sup from 26% β-mercaptoethanol ppt	0.754	29	30
4. DEAE cellulose chromatography	0.547	26	36
5. Agarose (0.5 M) chromatography	0.459	21	36

(3) The unmodified form of P_{II}, P_{IIA}, was crystallized in the cubic space group I23 (with Dr. Sae W. Suh). The unit cell dimension is a = 88.8 Å, with six molecules in the unit cell. The molecule has the symmetry 222; i.e., four subunits in P_{IIA} assume tetrahedral structure.

(4) UV spectra of P_{IIA}, P_{IID} and their difference spectrum were obtained. The difference spectrum is essentially the same as that of UMP. Using the second derivative spectrum of P_{IIA} in 6 M guanidine, protein concentrations and aromatic amino acid contents could be quantitated. The ratio of phenylalanine to tyrosine to tryptophan was 4.8:2.0:0.06. This result is in good agreement with the result of amino acid analysis which shows that P_{II} contains five phenylalanine and two tyrosine, but no tryptophan. Assuming that the extinction coefficient of the UMP moiety is not changed when bound to P_{II}, an empirical formula for calculating the state of uridylylation and protein concentration could be derived. The calculation requires solving two simultaneous equations:

$$A_{260} = 1.995\ (A + D) + 9.9\ OD + 2.92\ A_{340}$$

$$A_{290} = 0.7011\ (A + D) + 0.277\ D + 2.17\ A_{340}$$

where A and D represent the subunit concentration (mM) of P_{IIA} and P_{IID}, respectively.

(5) Amino acid sequence of a tryptic undecapeptide containing covalently bound UMP. The heat denatured $P_{II}[^3H, ^{32}P\text{-UMP}]_4$ was treated with trypsin and a peptide peak containing 91% of total radioactivity was purified from HPLC. The peptide was found to consist of eleven amino acids and to contain an uridylylated tyrosine, as shown below:

Gly-Ala-Glu-Tyr-Met-Val-Asp-Phe-Leu-Pro-Lys
|
UMP

(6) Properties of phosphotyrosly P_{II}. The phosphotyrosyl P_{II} was prepared by incubating $[^3H, ^{32}P]$uridylylated P_{II} with micrococcal nuclease. During the entire time course of incubation, P_{IID} activity decreased in parallel with the tritium labeled protein in TCA precipitates, and P_{IIA} activity increased in parallel with the appearance of $[^3H]$uridine in TCA supernatants, while ^{32}P radioactivity associated with the TCA precipitates remained constant. The P_{IIA} activity associated with the nuclease reaction mixture at the completion of the reaction was only 2% of the P_{IIA} activity expected for unmodified P_{II} at equivalent concentration. These results indicate that indeed phosphotyrosyl P_{II} was generated and the phosphatyrosyl P_{II} does not activate the deadenylylation reaction catalyzed by adenylyltransferase.

(7) A new fluorometric assay for the deadenylylation reaction was developed. To develop the continuous assay, an extrinsic fluor is introduced into the adenylylated glutamine synthetase by adenylylating the enzyme with 2-aza-1,N^6-etheno-ATP (Aza-ε-ATP), a fluorescence analog of ATP. When the modified enzyme (Aza-ε-GS) was deadenylylated with adenylyltransferase in the presence of P_{IID}, the amplitude of fluorescence monitored at the excitation wavelength of 300 nm and emission wavelength of 470 nm decreased by 40%. The fluorescence decrease due to the deadenylylation was linear with respect to time up to 70% completion of the reaction when saturated concentration of α-ketoglutarate was used, and the initial rate was linearly dependent on the concentration of either adenylyl-

transferase or P_{IID}.

(8) Monoclonal antibodies specific to various antigenic determinants of glutamine synthetase from E. coli were prepared. Antibodies specific to adenylyl moiety or specific to a fluorescent analog of AMP, aza-ε-AMP, were obtained by immunizing BalbC mice with AMP-BSA or aza-ε-AMP-BSA conjugate. The antigen-primed spleen cells from the immunized mice were hybridized with SP2 myeloma cells and screened for cells producing immunoglobulin which bind adenylylated glutamine synthetase or aza-ε-GS. Four clones producing different subclasses (IgM, IgG1, $IgG2_a$, and IgG3) were selected for AMP specific immunoglobulins and two clones producing IgG1 and $IgG2_b$ for aza-ε-AMP specific immunoglobulins.

Antibodies recognizing antigenic determinants other than adenylyl moiety were also obtained from mice immunized with E. coli glutamine synthetase. Seven clones which produce antibodies with different properties were selected. Preliminary characterization of unpurified antibodies by the use of ELISA assay indicated that two monoclonal antibodies (IgM, IgG1) clearly distinguish the relaxed form of glutamine synthetase from the taut form, presumably by binding to subunit interaction site. One clone produced antibody which inhibits the glutamine synthetase catalyzed γ-glutamyltransferase activity while the other six did not. In order to prepare a large quantity of antibody, cloned cells were injected into the peritoneal cavity of mice, ascites fluids were collected and subsequently immunoglobulins were purified by fractional salting-out, followied by DEAE-cellulose column chromatography and gel filtration. Further purification and quantitative characterization studies are in progress.

Proposed Course of Research

1. Various restriction enzyme generated DNA fragments from pglnD1 will be purified from agarose gel and ligated to a cloning vector CPBR 322 or PBR 325). Once we obtain a smaller fragment carrying the glnD gene, it will be moved to a cloning vehicle carrying a strong promoter such as the λ phage promoter PL.

2. Anti-UMP antibody-immunoadsorbant gel will be prepared to separate P_{II} depending upon the number of uridylylated subunits per tetramer.

3. Monoclonal antibodies will be further characterized.

Publications

Rhee, S. G., Ubom, G. A., Hunt, J. B, and Chock, P. B.: Catalytic cycle of the biosynthetic reaction catalyzed by adenylylated glutamine synthetase from E. coli. J. Biol. Chem. 257: 289-297, 1982

Huang, C. Y., Rhee, S. G., and Chock, P. B.: Subunit cooperation and enzymatic catalyses. Ann. Rev. Biochem. 51: 935-971, 1982.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00240-03 LB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Purification and Characterization of Selenium-containing Transfer RNAs

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Wei-Mei Ching	Staff Fellow	LB NHLBI
Other:	Thressa C. Stadtman	Chief, Section on Intermediary Metabolism and Bioenergetics	LB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Intermediary Metabolism and Bioenergetics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A selenium-containing tRNA from C. sticklandii was isolated and identified as a glutamate accepting tRNA. There is one atom of selenium per active tRNA-Glu molecule. The presence of the selenonucleoside (mam-5-Se-2-U) in this tRNA-Glu is essential for its acylation with glutamate. Preliminary results indicate that seleno-tRNAs may also exist in mammalian cells.

Progress

A. Seleno-tRNAs from anaerobic bacteria

1. Seleno-tRNAs from Clostridium sticklandii were enriched by affinity chromatography on an Hg-column. In the enriched sample, more than 20% of the total population contained selenium. One of these seleno-tRNAs was isolated as glu-tRNA^{Glu} by reversed-phase chromatography following the Hg-column enrichment. The most likely candidates for the other seleno-tRNAs are gln, lys and trp isoacceptors.

2. Characterization of seleno-tRNA^{Glu} from C. sticklandii:

a) The major isoaccepting tRNA^{Glu} in C. sticklandii is a seleno-tRNA. There is one atom of selenium per active tRNA^{Glu} molecule.

b) The presence of the selenium in this tRNA^{Glu} is essential for its enzymic acylation with glutamate. This was demonstrated by (i) the parallel loss of selenium content and charging activity during deacylation treatment and (ii) the separation of active species from the partially de-selenized mixture by Hg-column treatment.

c) This Se-tRNA^{Glu} recognizes both GAG and GAA codons in ribosome binding assays. There is no indication of preference between these two codons but it does not respond to the wrong codons (UAG, UAA).

d) The seleno-tRNAs from C. sticklandii have a common seleno base: mam^{5}Se^{2}U. This is consistent with the following observations: (i) the elution profile of the seleno-nucleoside on HPLC, (ii) the spectrum of the seleno-nucleoside separated from enzymic digests of seleno-tRNA^{Glu}, (iii) the difference spectrum of the purified seleno-tRNA^{Glu} vs. the same sample after partial de-selenization. There are more than one selenium modified base in the bulk tRNA of Methanococcus vannielii, but the major one is the same as in C. sticklandii.

3. Comparison of the major Se-tRNA^{Glu} with the minor glutamate isoaccepting species (tRNA$_2^{Glu}$) may elucidate the biological significance of seleno-tRNAs. A preliminary experiment indicated that the seleno-tRNA^{Glu} has higher affinity for glutamyl-tRNA synthetase than tRNA$_2^{Glu}$. This minor tRNA$_2^{Glu}$ was enriched in the 0.1-0.2 N NaCl wash of the Hg-column and partially purified after 2 runs on a RPC-5 column.

B. Seleno-tRNAs from mammalian cells:

Preliminary results indicate that seleno-tRNAs might be natural components of tRNAs from mouse leukemia cells (L1210), mouse liver, kidney, spleen and Ehrlich acites tumor cells. Selenium containing tRNAs from L1210 cells were resolved into two species on a RPC-5 column. Both of them eluted at $\sim$0.8 M NaCl indicating a very hydrophobic nature.

Proposed Action:

1. Sequence analysis: Information of the location of the seleno-base in the primary structure of seleno-tRNA^{Glu} may shed light on the functional role of this modified base. Comparison of the sequence of seleno-tRNA^{Glu} with tRNA$_2^{Glu}$ (after being purified, see below) will indicate the origin of seleno-tRNAs: are the isoacceptors transcribed from different genes or is one the result of modification of the other isoacceptor. If the second case is true, the de-selenized tRNA^{Glu} can serve as the substrate to search for the modifying enzyme(s).

2. Develop other methods for tRNA purification: since $tRNA_2^{Glu}$ is only partially purified by RPC-5 chromatography and other seleno-tRNAs from C. sticklandii and mammalian cells are unstable on this reversed phase column, other methods of purifying tRNAs are needed for further studies. Purification in the form of the tight complex between the amino acyl-tRNA, elongation factor Tu and GTP, may prove to be useful. Also two dimensional gel electrophoresis and reversed-phase boronate chromatography may be employed to purify tRNAs of interest.

3. Variations in patterns of isoaccepting tRNA species have been noted in differentiating cells, tumor cells, different organs and in response to carcinogens and hormones. To see if seleno-tRNAs are involved in cell transformation, the two very hydrophobic seleno-tRNAs from L1210 cells will be further characterized and compared with their counterparts in normal tissues.

Publications

Ching, Wei-Mei, and Stadtman, T.C.: Selenium containing $tRNA^{Glu}$ from Clostridium sticklandii: Correlation of aminoacylation with selenium content. Proc. Natl. Acad. Sci. USA 79, 374-377 (1982).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00241-03 LB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Detection of Cellular Proteins Containing Tyrosine Residues Modified by Phosphate or Nucleotide Phosphate

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.: Todd Martensen Guest Worker LB NHLBI

COOPERATING UNITS (if any)
None

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Section on Enzymes

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NHLBI, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A search for enzymatic activities in cells which hydrolyze protein phosphotyrosine residues was carried out utilizing a novel phosphotyrosyl protein substrate, phosphotyrosyl glutamine synthetase. Data suggest that alkaline phosphatase activities in cells do not play a role in the regulation of the phosphotyrosine content of proteins. A potent protein phosphotyrosyl phosphatase (PTPase) activity in both normal cells and malignant cells was observed. The PTPase activity in Ehrlich Ascites Tumor cells has been semipurified and found to elute as a single species on DEAE cellulose chromatography. This activity appears to be specific for proteins and distinct from protein phosphatase activities which dephosphorylate phosphoserine and phosphothreonine residues.

PHS-6040
(Rev. 2-81)

Project Description

Protein phosphorylation is a fundamental mechanism for regulating enzymatic activity. Phosphorylation of protein tyrosyl residues has been shown to be closely linked to retrovirus induced cell transformation, and the action of several growth factors. The identity of only a few cellular target proteins for several protein tyrosyl kinases has been accomplished. The regulation of these protein tyrosyl kinases, and the role of phosphoprotein phosphatases in regulating the turnover of the phosphotyrosyl bond in proteins, is poorly understood. E. coli adenylylated glutamine synthetase provides a most important tool in the development of methodologies to study the identification and quantitation of Tyr-P in proteins, the isolation of phosphotyrosyl proteins, and the assay and regulation of phosphotyrosyl protein phosphatase activity in normal and transformed cells.

Nucleotide linked to protein through a phosphodiester bond to tyrosine exists rarely in nature. Enzymatic activity which unlinks the polynucleotide of polio virus attached to a viral protein has been found in HeLa cells. Any relationship between protein Tyr-P and protein tyrosine nucleotidylyl phosphodiester remains unknown. Development of specific assays, and antibodies to nucleotidylyl proteins is necessary for these investigations.

Major Findings

1. A potent PTPase activity exists in cells which may play a significant role in regulating the level of phosphotyrosine in proteins.

2. Alkaline phosphatase poorly catalyzes the hydrolysis of protein phosphotyrosyl residues. In cells in which alkaline phosphatase activity is induced > 100-fold that of basal levels, no corresponding increase in PTPase was observed.

3. IgG which recognizes Tyr-P inhibits phosphatase degradation of Tyr-P. This may be important in enabling one to assay the low levels of tyrosine kinase in normal cells.

4. Ehrlich Ascites Tumor cells contain protein inhibitors of PTPase activity.

Significance to Biomedical Research

Detection of phosphotyrosine in proteins and identification of specific tyrosine kinase(s) and protein phosphotyrosine phosphatase activities which regulate its level will be necessary to understand how phosphorylation of protein tyrosine residues is linked to growth, especially uncontrolled growth of cells.

Proposed Course of Research

Detection and isolation of proteins containing Tyr-P in cells. Development of antisera to nucleotidylyl tyrosine. Utilization of radiolabeled phosphotyrosyl and adenylyl glutamine synthetase to try to detect protein phosphotyrosyl phosphatase and tyrosyl nucleotide diesterase in cells.

Publications

Martensen, T. M.: Phosphotyrosine in proteins: Stability and quantification. J. Biol. Chem., in press, 1982.

Martensen, T. and Levine, R.: Base hydrolysis of proteins and amino acid analysis for tyrosine phosphate. Meth. Enzymol., in press, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00242-02-LB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Biosynthesis and Properties of Selenium-containing tRNA from Escherichia coli.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Arthur J. Wittwer	Staff Fellow	LB NHLBI
Other:	Thressa C. Stadtman	Chief, Section on Intermediary Metabolism and Bioenergetics	LB NHLBI

COOPERATING UNITS (if any)

None

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NHLBI, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Escherichia coli specifically incorporate selenium into tRNA. Forty percent of the selenium-containing tRNA had lysine acceptor activity and an additional 10% had glutamate acceptor activity. About 5% of the total tRNA-lys and 1% of the total tRNA-glu was selenium-modified. Analysis by high performance liquid chromatography (HPLC) indicated the presence of only one selenium-containing nucleoside. The UV absorbance spectrum and elution position during HPLC of this selenonucleoside identify it as 5-methylaminomethyl-2-selenouridine, a previously uncharacterized compound.

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Project Description

From previous work it was known that Escherichia coli synthesize selenium-containing tRNA. The amount of selenium-containing tRNA was neither decreased by excess sulfur compounds, increased by excess selenium, nor blocked in mutants unable to synthesize 4-thiouracil. This implied a specific incorporation mechanism and a potential physiological importance for selenium modification. In hopes of understanding the biochemical role of selenium-modified tRNA in E. coli, the present work sought answers to two questions:

1. What is the amino acid acceptor specificity of the selenium-containing tRNA species?

2. What is the structure of the selenium-modified nucleoside or nucleosides?

Major Findings

(1) Amino acid acceptor specificity. Elution position during RPC-5 chromatography was used to indicate the aminoacylation status of different radioactively labeled tRNA species. Elution profiles of [^{75}Se]tRNA, isolated under conditions which preserved in vivo aminoacylation, differed markedly from the patterns observed with parallel preparations that had been deacylated at pH 8 prior to chromatography. Subsequent enzymatic acylation with an amino acid mixture restored the original elution pattern indicative of the in vivo state. This showed that the [^{75}Se]tRNA was both aminoacylated in vivo and could be aminoacylated in vitro. Aminoacylation of the deacylated [^{75}Se]tRNA preparation with lysine alone shifted the elution position of a major selenium-containing tRNA (40% of the ^{75}Se radioactivity). The elution position was not affected when the tRNA was incubated with the aminoacylation reaction mixture in the absence of lysine. Similar experiments showed that another 10% of the selenium-containing tRNA could be aminoacylated with glutamate, as indicated by an analogous shift in elution position.

These experiments showed that about one-half of the selenium-containing tRNA consisted of lysine- and glutamate-accepting species. The amino acid acceptor specificity of the remaining seleno-tRNA has not been determined. Quantitation with [^{14}C]lysine and [^{14}C]glutamate indicated that about 5% of the total $tRNA^{lys}$ and 1% of the total $tRNA^{glu}$ was modified with selenium.

(2) Characterization of the selenium-containing nucleoside. Analysis of enzymatic digests of bulk [^{75}Se]tRNA by reversed-phase high performance liquid chromatography (HPLC) indicated the presence of a single radioactively labeled peak. When phosphatase was omitted during the nuclease digestion the elution position shifted to an earlier time, confirming the nucleoside nature of the selenium-containing moiety.

Selenium-enriched [^{75}Se]tRNA (386 pmol Se/A_{260} unit) was prepared by successive chromatography on Sepharose-4B and RPC-5. Digestion and HPLC of this material allowed the isolation of apparently pure selenonucleoside. This material was tentatively identified as 5-methylaminomethyl-2-selenouridine on the basis of the following observations:

a) The selenonucleoside has UV absorbance maxima characteristic of a 2-selenouridine derivative. Its spectrum is qualitatively dissimilar to the spectra given by 4-selenouridine, 6-selenoguanosine, and 2-selenocytidine, which are the other possible selenium analogues (Se replacing O) of the common ribonucleotides.

b) The UV absorbance spectrum is qualitatively similar to that of 5-methylaminomethyl-2-thiouridine, except that it is shifted to higher wavelengths, a characteristic of seleno- vs thio-substitution. In particular, the spectra are similar in the portions thought to be contributed by the 5-methylaminomethyl side chain.

c) 5-methylaminomethyl-2-thiouridine has been identified in E. coli $tRNA^{lys}$ and $tRNA^{glu}$ as a minor nucleoside. It is not uncommon for biological selenium compounds to be analogues of naturally occurring sulfur-containing molecules.

d) The elution position of the selenonucleoside during HPLC was found to be about 1 min after 5-methylaminomethyl-2-thiouridine. The elution position of 5-methyl-2-selenouridine was similarly displaced from that of 5-methyl-2-thiouridine.

Synthesis of 5-methylaminomethyl-2-selenouridine, a previously uncharacterized compound, was accomplished by Dr. L. Tsai of this laboratory. The UV spectrum and elution position during HPLC appeared to be identical to the natural selenonucleoside, thus confirming the identity of the unknown compound.

Proposed Course of Action

(1) Purify the selenium-containing $tRNA^{lys}$ and/or $tRNA^{glu}$ from E. coli and determine its sequence - - particularly the location of the 5-methylaminomethyl-2-selenouridyl residue. Ribosome binding studies to determine codon specificity should also be performed.

(2) Investigate the enzymology of selenium modification. It is of great interest to know the identity of the selenium donor in this reaction.

Publications

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00243-02 LB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Analysis of an Inactivated Form of the Glutamine Synthetase of E. coli

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Joshua Farber	Research Associate (Senior Staff Fellow)	LB NHLBI
Other:	Rodney L. Levine	Research Associate (Senior Surgeon, USPHS) (Medical Staff Fellow as of 6/27/82)	LB NHLBI

COOPERATING UNITS (if any)

None

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Section on Enzymes

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NHLBI, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Previous work in this laboratory has shown that during bacterial growth under conditions in which glutamine synthetase (GS) activity decreases, antigenically cross-reacting material initially persists, suggesting that inactivation precedes degradation. Examination of the inactivating activity of cell-free extracts led to the development of a model inactivating system including only ascorbic acid, iron, and oxygen as necessary components. This and similar systems have been shown in this laboratory to inactivate a number of enzymes. Acid hydrolysis and amino acid analysis of the inactivated GS revealed the loss of one histidine residue per subunit as compared to the native control. It was undertaken to identify and isolate from the inactivated enzyme a peptide containing the altered histidine. A bacterial strain was constructed which overproduced GS allowed for the specific radiochemical labeling of the histidine residues so that the histidine containing peptides could be identified and the fate of the altered histidine traced. Trypsin and cyanogen bromide peptides of the native and inactivated GS were separated using reverse phase chromatography. A small, hydrophilic cyanogen bromide peptide from the inactivated enzyme has been tentatively identified which on amino acid analysis lacks one histidine compared to the corresponding peptides for the native enzyme.

Project Description

Previous work in this laboratory has shown that during bacterial growth in conditions under which GS activity is being lost, antigenically cross-reacting material initially persists, suggesting that inactivation precedes degradation. Examination of the inactivating activity of cell-free extracts led to the development of a model inactivating system including only ascorbic acid, iron, and oxygen as necessary components. This and related systems have been shown in this laboratory to inactivate a number of enzymes. The characteriestics of the inactivating system suggest similarities to the oxygen mediated bacteriocidal activity of leukocytes. Thus, in addition to its possible role in proteolysis, this type of enzyme inactivation may be involved in leukocyte killing of bacteria. Initial amino acid analysis of GS suggested the loss of one histidine residue per subunit in the inactivated enzyme. It is of interest to determine the nature of the structural modification in the inactivated enzyme. The goal of this project has been to produce and isolate a peptide which contains the amino acid modification responsible for inactivation. Identification of such a peptide would provide assurance that the modification of a histidine was not dependent on acid hydrolysis of the protein, provide material suitable for chemical identification of the modified amino acid, and perhaps allow for the production of an antibody specific to the modification. Analysis of the peptide would clarify the mechanism of inactivation and enable comparison of products of *in vitro* inactivating systems as well as *in vivo* isolates.

Major Findings

(1) Dr. Emilio Garcia in this laboratory transferred the plasmid from an *E. coli* strain which overproduces GS (from the laboratory of Dr. Boris Magasanik) to a new background strain which when grown in the presence of radioactive histidine produced GS labeled only in the histidine residue. This provided a powerful tool for evaluation of the histidine modification.

(2) Using [^{14}C]histidine GS inactivated with ascorbic acid and [^{3}H]histidine GS as the control, the modification of one histidine residue in the inactivated GS was confirmed. It was found that acid hydrolysis converts one histidine in the inactivated enzyme to multiple products, one of which is the Fluoropa positive peak identified in the amino acid analyzer as peculiar to the inactivated enzyme.

(3) The [^{14}C]histidine and [^{3}H]histidine labeled GS, as well as unlabeled enzyme were used in an attempt to identify and isolate a peptide containing the modified histidine residue. Analysis of cyanogen bromide peptides using HPLC reverse phase chromatography initially failed to identify a peptide from the inactivated protein with a modified histidine, nor could we identify its parent in the peptide of the native enzyme. However, reduction and carboxymethylation of the proteins either before or after cyanogen bromide cleavage revealed a small, hydrophilic cyanogen bromide fragment which in the case of the inactivated enzyme showed the loss of a histidine residue. Final purification and further characterization of the peptide is ongoing.

(4) We have pursued observations described in last year's report on the increased susceptibility of the inactivated GS to proteolytic attack by subtilisin. The rate of initial cleavage of both the native and inactivated enzymes is slowed in the presence of magnesium compared with manganese, divalent cations being known to bind avidly to GS. The possibility that differences in the rates of proteolysis

of the two forms of the protein result from differences in their metal binding properties is being investigated

Significance to Biomedical Research

Protein turnover is a basic biological process. Oxidative inactivation of glutamine synthetase has been implicated as a preliminary step in the turnover of this enzyme in bacteria. It also may play a role in the oxygen mediated leukocyte killing of bacteria. Structural analysis of an oxidatively inactivated form of GS will shed light on the mechanism of inactivation and possibly on a key factor in the control of intracellular protein degradation. Information on the control of protein degradation is of general biomedical interest and recently of particular concern regarding the prevention of the unwanted degradation of the products of cloned genetic material. The precise mechanism of leukocyte mediated bacterial killing of activated oxygen species is unknown as are the crucial intracellular targets. Knowledge of the mechanism of oxidative inactivation of GS and other bacterial enzymes may help in the understanding of this biological phenomenon. In addition, information regarding the mechanism of inactivation will shed light on the mechanism of the synthetase's catalysis. The study of subtilisin mediated proteolysis of native and modified GS will yield information regarding structural aspects of the two proteins and their interactions with ligands, as well as a more detailed analysis of a proteolytic process. Such processes are widely represented in physiological/pathological phenomena such as intracellular and extracellular protein processing, coagulation, complement activation, and tissue injury.

Proposed Course of Research

Research in the immediate future will concentrate primarily on a structural analysis of the oxidatively inactivated GS, and in particular, on the purification and characterization of the altered peptide and its modified histidine. This will be done with a view toward evaluation of the possible role of such a modification in the biological processes of protein degradation and leukocyte killing. Additional aspects of the interaction between subtilisin and the native and inactivate GS will also be investigated.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00244-02 LB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Oxidative Inactivation of Enzymes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Laura Fucci-Laccetti	Visiting Fellow	LB NHLBI
Others:	E. R. Stadtman	Chief, Laboratory of Biochemistry	LB NHLBI
	Cynthia N. Oliver	Staff Fellow	LB NHLBI

COOPERATING UNITS (if any)

None

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We showed previously that E. coli glutamine synthetase (GS) is inactivated in vitro by several mixed function oxidation systems. The inactivation requires O_2, is stimulated by Fe^{+3} and is inhibited by catalase. Nitrogen starvation provokes a similar inactivation of GS in E. coli in situ. In order to test the generality of this phenomenon, we studied the capacity of a microbial NADH-diaphorase system and a rabbit liver microsomal P450 system to inactivate enzymes other than GS. Of the nineteen additional enzymes tested, the diaphorase system inactivated alcohol dehydrogenase, aspartokinase III, creatine kinase, glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, phosphoglycerate kinase, and pyruvate kinase. Except for aspartokinase III, not yet tested, the same enzymes were inactivated by the P450 system. Participation of hydrogen peroxide as an intermediate in these inactivation reactions is inferred by catalase inhibition. Fe^{+3} is reduced to Fe^{+2} by diaphorase and cytochrome P450 systems. In addition to in vitro inactivation studies, experiments were undertaken in E. coli K12 cells in order to study the possible role of this inactivation in vivo. Techniques used in these studies have included polyacrylamide gel electrophoresis, high pressure liquid chromatography, amino acid analysis, chromatographic techniques and enzymatic assay of functional proteins.

Project Description

Many researchers in our laboratory are involved in a study of the regulation of the enzyme levels in the cells. The intracellular enzyme levels reflect the balance between synthesis and degradation. Previous studies in our laboratory suggested that GS degradation is a two-step process involving oxidative inactivation followed by proteolysis. The present report is concerned with the generality of the initial step, the oxidative inactivation.

Major Findings

From in vivo and in vitro experiments (Levine et al., Proc. Natl. Acad. Sci. U.S. 78, 2120-2124, 1981; and Oliver et al., in Ornston, L. N. (ed.): Experiences in Biochemical Perceptions, New York, Academic Press, pp. 233-249), there is evidence that glutamine synthetase (GS) modified by oxidative inactivation is more susceptible to proteolytic degradation. Several enzymic systems (rabbit microsomal cytochrome P450 mixed function oxidation system, microbial diaphorase system) and several nonenzymic systems (ascorbate, Fe^{+2}) are capable of oxidatively inactivating GS with the qualitatively similar characteristics.

In order to test the generality of this phenomenon, we checked the capacity of the diaphorase system to inactivate the enzymes shown in Table I. Of the nineteen different enzymes, the diaphorase system inactivated alcohol dehydrogenase (ADH) from yeast and L. mesenteroides, aspartokinase III from E. coli (AK), phosphoglycerate kinase from yeast (PGK), creatine kinase (CK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), lactate dehydrogenase (LDH) and pyruvate kinase (PK) from rabbit muscle. These enzymes, except for AK (not yet tested), were inactivated also by the cytochrome P450 system. Several of the enzymes that were

Enzyme	Source
Acetate kinase	E. coli
Alcohol dehydrogenase	L. mesenteroides
	Yeast
Aldolase	Rabbit muscle
Alkaline phosphatase	E. coli
α-Amylase	Porcine pancreas
Aspartokinase III	E. coli
Carboxipeptidase A	Bovine pancreas
Creatine kinase	Rabbit muscle
Fructose-1,6-diphosphatase	Rabbit muscle
β-Galactosides	E. coli
Glucose-6-phosphate dehydrogenase	L. mesenteroides
	Baker's yeast
β-Glucuronidase	E. coli
Glyceraldehydro-3-phosphate dehydrogenase	Rabbit muscle
Hexokinase	Yeast
Lactate dehydrogenase	Rabbit muscle
Lysozyme	Hen egg white
Malic dehydrogenase	Bovine heart
	Porcine heart
Phosphoglycerate kinase	Yeast
Pyruvate kinase	Rabbit muscle

inactivated are known to have a histidine associated with the active site. Amino acid analysis of the NADH-diaphorase-inactivated PGK indicated that a single histidine per subunit was lost after inactivation (R. L. Levine). These results are similar to those observed with GS inactivation. These results suggested the possibility that the diaphorase system could catalyze a site specific oxidation of different enzymes by a mechanism similar to that already proposed for GS inactivation. Because many of the enzymes inactivated are dehydrogenases and kinases, the site of the inactivation could be the nucleotide binding site. This idea is supported by the observation that GS is protected from inactivation (NADH-diaphorase system) by ATP. In addition, we found that PK is protected by ADP and PKG is protected by ATP.

All the inactivation reactions studied required O_2 and were inhibited by catalase. Superoxide dismutase had no effect. EDTA and O-phenanthroline completely inhibited the NADH-diaphorase inactivation (except with the ADH). Because these data suggested the participation of H_2O_2 as intermediates in the inactivation reaction, we studied the effect of H_2O_2 alone. All the enzymes, except GS and PGK, were inactivated by H_2O_2 but at different rates. At least for the last two enzymes, we cannot explain the inactivation by diaphorase on the basis of H_2O_2 production alone. Diaphorase can produce H_2O_2 and can reduce Fe^{+3} to Fe^{+2}. In the GS inactivation by diaphorase, H_2O_2 seems not to be the limiting step because the addition of H_2O_2 (0.88 mM) to the complete NADH-diaphorase system does not stimulate the inactivation. However, addition of low concentrations of Fe^{+2} does stimulate the inactivation of GS by the complete NADH-diaphorase system.

Diaphorase and cytochrome P450 systems inactivated GS, PGK and PK with the same characteristics. The inactivations required O_2, were stimulated by Fe^{+3} and were inhibited by catalase, Mn^{+2}, EDTA and O-phenanthroline. These results suggested that the inactivation of these enzymes is a multistep process involving (a) reduction of iron III; (b) binding of iron II to divalent cation site of the enzymes; (c) *in situ* auto-oxidation of iron II to III with the generation of one or more activated oxygen species, and (d) *in situ* oxidation of a susceptible histidine which is associated with the active-site. In order to identify the oxygen species implicated in the GS inactivation by diaphorase, we tested the effect of radical scavengers such as mannitol, thiourea and histidine. Only histidine (100 mM) completely inhibited this inactivation. In order to study this inactivation *in vivo*, we determined the levels of PK, PGK, and GAPDH in *E. coli* K-12 cells under a variety of metabolic conditions. Preliminary results in the cells under starvation indicated that the level of PGK decreased and that of GAPDH increased, and that of PK was nearly constant. In the presence of chloramphenicol, the level of PK decreased, too. We observed slightly different results during glucose and nitrogen starvations.

Proposed Course of Research

Our studies will be concentrated on the further characterization of these oxidative inactivation reactions. We would like to determine if enzymes which are inactivated exhibit site specific oxidative modification of histidine or other residue. In addition, we hope to clarify the mechanism of the inactivation reactions, including the mode of H_2O_2 production, iron reduction, and their interaction with the target enzyme. We are attempting to study the inactivation (of one or two enzymes) *in situ* in *E. coli* and to study the physiology of this inac-

tivation under a variety of metabolic conditions. We plan to prepare specific antibody and use immunoprecipitation techniques in order to distinguish inactivation from proteolysis. With these techniques, it may possible to study the kinetics of these two processes.

Publications

Oliver, C., Fucci, L., Levine, R., Wittenberger, M., and Stadtman, E.R.: Inactivation of Key Metabolic Enzymes by P450-Linked Mixed Function Oxidation Systems. In Hietanen, E. (Ed.): Cytochrome P-450. Biochemistry, Biophysics and Environmental Implications. Amsterdam, Elsevier-North Holland, 1982, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00245-01 LB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Oxidative Inactivation of Glutamine Synthetase Subunits

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Kazuyuki Nakamura	Visiting Fellow	LB NHLBI
Other:	Earl R. Stadtman	Chief, Laboratory of Biochemistry	LB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
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SECTION
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NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.8	0.2

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Partial inactivation of glutamine synthetase (GS) by a mixed function oxidase model system composed of ascorbate, O_2 and Fe(III) leads to the formation of hybrid GS molecules (dodecamers) composed of both inactive and active subunits. Subunit interaction in these hybrid molecules are weaker than in the native enzyme, as is indicated by the kinetics of subunit dissociation in the presence of 4 M urea. Heterologous subunit interactions in these hybrid molecules do not affect the affinity of active subunits for glutamate. Incubation of partially adenylylated glutamine synthetase preparations ($\bar{n}$ = 6.7) with the ascorbate system in the absence of substrates leads to preferential oxidation (inactivation) of unadenylylated subunits, whereas incubation in the presence of ATP and glutamate leads to preferential inactivation of adenylylated subunits.

PHS-6040
(Rev. 2-81)

Project Description

Glutamine synthetase (GS) from Escherichia coli is inactivated by any one of several different mixed function oxidase systems, and by a nonenzymic system composed of ascorbate, O_2, and Fe(III). The inactivation reaction is regulated by the concentrations of ATP and glutamate and by the state of adenylylation, $\bar{n}$, of the enzyme. In absence of substrates, the unadenylylated enzyme is more susceptible to inactivation, whereas in the presence of ATP and glutamate, inactivation of unadenylylated enzyme is suppressed and the inactivation of the adenylylated enzyme is greatly accelerated. The present study was undertaken to determine if: (a) partial inactivation of a GS preparation by the ascorbate system leads to a mixture of fully inactivated and fully active GS molecules, or to a population of hybrid molecules (i.e., dodecamers composed of both active and inactive subunits; (b) partial inactivation of either fully adenylylated or unadenylylated GS affects subunit interactions of the dodecameric structure; and (c) substrates direct the inactivation of adenylylated vs unadenylylated subunits in GS molecules containing six subunits of each type (i.e., GS in which $\bar{n} = 6.0$). Goals (a) and (b) were examined by measuring the kinetics and extent of enzyme dissociation of partially inactivated GS in the presence of 4.0 M urea (c.f., J. Ciardi, F. Cimino, and E. R. Stadtman, Biochemistry 12, 4321, 1973). Goal (b) was further examined by determining the effect of partial inactivation on the kinetic parameters of the residual GS activity. Goal (c) was examined by measuring changes in the levels of catalytically active adenylylated and unadenylylated subunits present in partially adenylylated GS preparations ($\bar{n} = 6.7$) following partial inactivation of the enzymes by the ascorbate system in the presence and absence of ATP and glutamate. The ratio of adenylylated and unadenylylated GS subunits was determined by measuring the γ-glutamyltransferase activity in the presence of Mn^{2+} alone under conditions where both kinds of subunits are equally active (total activity), and again in the presence of Mg^{2+} when only the unadenylylated subunits are active. From these measurements, the apparent state of adenylylation, $\bar{n}$, can be calculated. It follows that if unadenylylated subunits are preferentially inactivated, the value of $\bar{n}$ will increase, whereas if the adenylylated subunits are preferentially inactivated, the value of $\bar{n}$ will decrease.

Major Findings

1. Partial inactivation of unadenylylated GS by the ascorbate system leads to the formation of hybrid molecules (dodecamers) composed of both active and inactive subunits. The partially inactivated enzyme is more easily dissociated by urea than is the native enzyme; this indicates that interactions between active and inactive subunits are weaker than homologous interactions of active subunits.

2. Fifty percent inactivation of unadenylylated GS by the ascorbate system has no effect on the K_m of the enzyme for glutamate. Thus, the K_m is not affected by heterologous interactions between active and inactive subunits.

3. Incubation of partially adenylylated preparations of GS ($\bar{n} = 6.7$) with ascorbate, O_2 and Fe(III) in the absence of substrates leads to a time-dependent increase in the value of $\bar{n}$, whereas in the presence of ATP and glutamate, the value of $\bar{n}$ decreases. It is thus apparent that in the absence of substrates, the unadenylylated subunits are preferentially inactivated, whereas in the presence of ATP and glutamate, the adenylylated subunits are preferentially inactivated.

Proposed Course of Research

1. This project has been essentially finished.

2. We have purified cytochrome P-450 and NADPH-cytochrome P-450 reductase from rabbit liver microsome. Using those materials, the characterization of oxidative inactivation of glutamine synthetase or other enzymes by cytochrome P-450 mixed function oxidase will be done.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00246-01 LB

PERIOD COVERED
October 1,1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Characterization of a Selenium-containing Thiolase from Clostridium kluyveri

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Mark Sliwkowski	Staff Fellow	LB NHLBI
Others:	Thressa C. Stadtman	Chief, Section on Intermediary Metabolism and Bioenergetics	LB NHLBI

COOPERATING UNITS (if any)

None

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0.85	0.65	0.2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The function of selenium in thiolase isolated from Clostridium kluyveri is being studied. Preliminary isoelectric focusing studies have revealed that the selenothiolase is a basic protein (pI 8.7-9.0) and this finding has led to a modification of the isolation procedure. Previously, the final purification step had been chromatography on Matrex Gel Green resulting in a two-fold increase in specific activity but with a 50% loss of total activity. Chromatography on carboxymethyl-Sephadex proved to be a more satisfactory step in that it gave a similar increase in specific activity with 96% recovery of the total activity. Since the selenium moiety of thiolase is selenomethionine, antibodies specific for selenomethionine might be useful as a structural probe, a means of rapid isolation of the enzyme, or in surveys from other organisms, etc. Accordingly, selenomethionine has been covalently coupled to bovine serum albumin, in order that sheep antibodies may be raised.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: Nearly all of the selenoproteins studied to date contain selenium in the form of selenocysteine. The thiolase from Clostridium kluyveri however, contains selenomethionine. The role of this unique amino acid residue is unknown. Whether or not selenomethionine is directly involved in catalysis or plays a structural role in the protein will be the subject of further study. Selenomethionine is considerably more stable than selenocysteine, in that it is not spontaneously oxidized or eliminated under normal aerobic conditions. Thus, the selenomethionine-containing thiolase may be a very good candidate for determining the mechanism by which selenium is incorporated into specific proteins.

Major Findings

1) The selenium-containing thiolase is a basic protein with an isoelectric point between pH 8.7-9.0.

2) An alternative final purification step using carboxymethyl-Sephadex, rather than Matrex Gel Green gives greater yields, with a similar increase in specific activity.

3) Selenomethionine was conjugated to bovine serum albumin resulting in 16 moles of selenomethionine per mole of BSA, as determined by amino acid analysis. Sheep antibodies are being raised against this conjugate and their reactivity with thiolase will be tested.

Proposed Course of Action

1) Currently selenomethionine is quantitated using an amino acid analyzer. Under normal run conditions, selenomethionine and leucine coelute. Modification of the solvent program allows for partial resolution of the amino acids. An alternative method for selenomethionine quantitation might be achieved using reversed-phase HPLC, with either pre- or post-column derivatization.

2) Kinetic studies will also be initiated in order to elucidate the mechanism, substrate specificity, and essential residues involved in catalysis.

3) Physical studies of the enzyme will be conducted including sedimentation velocity and equilibrium analysis.

4) Selenium-containing peptides, generated by proteolytic and chemical methods, will be isolated and characterized.

5) Antibodies raised to selenomethionine-BSA will be used with the selenium-containing thiolase as a structural probe and for surveys involving other organisms. Also, the antibodies may be used for an immunoaffinity column as a means for the rapid isolation of thiolase.

Publications

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00247-01 LB

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Proteolysis of Glutamine Synthetase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Jo Ellen Roseman	Guest Worker (Joint Program FAES/NIH: Johns Hopkins University)	LB NHLBI
Other:	Earl R. Stadtman	Chief, Laboratory of Biochemistry	LB NHLBI
	Rodney L. Levine	Senior Surgeon, U.S.P.H.S. Medical Staff Fellow as of 6/27/82	LB NHLBI

COOPERATING UNITS (if any)

None

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.2	0.3

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To elucidate the mechanism of intracellular protein turnover, we have been attempting to isolate from bacteria a system that will degrade normal cell proteins. Glutamine synthetase from E. coli is proteolyzed by bacterial cell extracts once it has been oxidatively modified by a model inactivating system consisting of ascorbate, iron and oxygen. The purpose of this project is to purify a proteolytic activity, presumably a protease, that will preferentially degrade the inactive versus the native glutamine synthetase. Starting from E. coli whole cell extracts, a proteolytic activity has been partially purified which degrades the inactive glutamine synthetase 10 times more rapidly than the native. The proteolytic activity is soluble rather than membrane associated, precipitates in a 60-70% ammonium sulfate cut, does not bind DEAE ion exchanger at pH 7.5, is retained by an ultrafiltration membrane of 50,000 molecular weight pore size, and elutes ahead of the protein peak on gel filtration columns.

PHS-6040
(Rev. 2-81)

Project Description

Introduction

To elucidate the mechanism of intracellular protein turnover, we have been attempting to isolate from bacteria a system that will degrade normal cell proteins. Studies originally done in 1977 showed that glutamine synthetase activity is lost in nitrogen-starved bacterial cells prior to loss of glutamine synthetase cross-reacting protein. This led to the hypothesis that a modification which results in inactivation provides a signal to the proteolytic machinery of the cell. The inactivating activity found in bacterial extracts can be mimicked by a model system, consisting of iron, oxygen, and ascorbic acid. E. coli extracts preferentially degrade purified, ascorbate-modified glutamine synthetase as compared to native glutamine synthetase. The ascorbate-modified glutamine synthetase has been shown to be similar to native glutamine synthetase: it comigrates on native and SDS polyacrylamide gels, has the same fluorescence emission spectrum and shows only subtle differences in its UV absorption spectrum. The ascorbate inactivated glutamine synthetase shows the loss of a single histidine residue per 50,000 molecular weight subunit; otherwise, the amino composition of the two are identical.

Objectives

The goal of this project is to purify a proteolytic activity that preferentially degrades the ascorbate-modified glutamine synthetase over the native, presumably by recognition of the subtle and specific modification imparted by ascorbate treatment.

Methods Employed

The approach we have taken is to use a known physiologic substrate and try to find an E. coli protease that will degrade it. By growing cells on radioactive nutrients and allowing them to incorporate the nutrients into protein, we have produced radioactive protein that is unmodified by isotopic labelling techniques.

Glutamine synthetase was purified by the zinc precipitation method from an overproducing E. coli K_{12} strain grown on radioactive amino acids. The purified glutamine synthetase was of high enzymatic specific activity and was uniformly labeled with ^{14}C amino acids. A portion of this native enzyme was inactivated by the ascorbate model-inactivating system.

The radioactive glutamine synthetase is insoluble in trichloroacetic acid, and we developed an assay to measure the production of trichloroacetic acid soluble radioactivity as a result of protease treatment.

Major Findings

Starting from E. coli whole cell extracts, a proteolytic activity has been partially purified which degrades the inactive glutamine synthetase 10 times more rapidly than the native. The proteolytic activity is soluble rather than membrane associated, precipitates in a 60-70% ammonium sulfate cut, does not bind to DEAE ion exchanger at pH 7.5, is retained by an ultrafiltration membrane of 50,000 molecular weight pore size, and elutes ahead of the protein peak on gel filtration columns. The proteolytic activity which is being purified shows a ten fold preference for the inactive glutamine synthetase and has been purified about 1500 fold.

Proposed Course of Research

Further purification will be carried out. The purified protease will then be studied for its ability to degrade glutamine synthetase as well as other enzymes which can be oxidatively inactivated. It will also be used as a tool to find and purify the inactivating activity from *E. coli* extracts.

Publications

None.

ANNUAL REPORT OF THE CARDIOLOGY BRANCH,
National Heart, Lung, and Blood Institute
October 1, 1981 through September 30, 1982

The areas of experimental interest of the Cardiology Branch relate mainly to the pathophysiology and treatment of coronary artery disease, angina pectoris, and hypertrophic cardiomyopathy; to the evaluation and treatment of valvular heart disease; and to the development of noninvasive techniques to assess cardiac structure and function. We have continued to pursue the major directions of research launched two years ago; namely, defining 1) the role of calcium channel blocking drugs in the treatment of coronary artery disease and of hypertrophic cardiomyopathy, 2) the mechanisms of action responsible for the beneficial effects of these drugs and 3) the conditions leading to and clinical significance of abnormalities in left ventricular diastolic function. The major new area of investigation begun this year relates to the definition and clinical significance of changes in coronary vasoconstrictor tone as a cause of angina pectoris.

CALCIUM CHANNEL BLOCKING DRUGS

Calcium Channel Blocking Drugs in the Treatment of Hypertrophic Cardiomyopathy (HCM)

Several years ago we hypothesized that myocardial intracellular calcium overload may be a possible etiologic mechanism responsible for the hemodynamic abnormalities of pts with HCM. We subsequently demonstrated that verapamil decreased LV outflow obstruction, increased exercise capacity, and improved symptomatic status. Last year we explored the mechanisms responsible for those beneficial effects and found that verapamil reduced left ventricular outflow tract gradient and improved LV diastolic filling parameters. Additional studies demonstrated these two potentially clinically benefical actions were associated with an increase in both LV diastolic and systolic dimensions, as well as a decrease in LV ejection fractic It is believed that obstruction to left ventricular outflow is caused by Venturi forces impelling the anterior mitral valve leaflet toward the ventricular septum and into the outflow tract during systole. The changes induced by verapamil would attenuate these forces, since their magnitude is related to the velocity of blood coursing through the outflow tract. Thus, the improved LV filling caused by verapamil probably leads to the observed increase in LV volume, which would increase the narrowed LV outflow tract found in HCM. The larger outflow tract, in association with the drug-induced decrease in contractile state, would diminish the velocity of blood flow through the outflow tract and thereby reduce the Venturi forces. The decrease in gradient would diminish LV systolic pressure and thereby contribute to improved symptoms. In addition, the improved LV diastolic filling could lead to reductions in LV filling pressures, thereby ameliorating symptoms resulting from pulmonary congestion. In more detailed studies completed this year, we investigated the effects of verapamil on LV pressure volume loops. We found that when verapamil improves peak filling rate, LV end diastolic pressure decreases and the rate of LV relaxation increases. In addition, the pressure volume curve is shifted downward to the right throughout diastole, so that there is a lower LV diastolic pressure for any given diastolic volume. Thus, verapamil-induced improvement in LV rapid diastolic filling is associated with and probably caused by an enhancement of LV relaxation and LV compliance.

Relative effects of verapamil and nifedipine: Several calcium channel blocking agents are available for clinical use. Although each of these agents inhibits voltage dependent calcium channels, they are not identical in the spectrum of their physiologic actions, having different effects and potencies on different tissues. Preliminary data last year suggested that nifedipine does not have the same beneficial effects in patients with HCM as does verapamil. This year we continued those studies. Nifedipine was administered to 27 HCM pts at catheterization. Although nifedipine increased cardiac output and LV diastolic volume, it did not change LV outflow tract obstruction. In contrast to the decrease in peak filling rate produced by verapamil, nifedipine had no such action. The lack of substantial effect of nifedipine on LV systolic pressure or diastolic filling rate raised questions as to the potential efficacy of nifedpine in symptomatic control of pts with HCM. To assess this issue, we evaluated the relative effects of verapamil, nifedipine and placebo on exercise capacity and symptomatic status in pts with HCM. Drugs were given in a randomized double blind fashion in low and high doses. Our findings indicated that although nifedipine was helpful in some pts, verapamil more effectively improved exercise capacity and symptomatic status.

Calcium Channel Blocking Agents in the Treatment of Coronary Artery Disease (CAD): Over the past two years we demonstrated that verapamil is an effective antianginal agent in pts with incapacitating symptoms due to CAD. We also demonstrated that 90% of all pts with CAD manifest abnormalities in LV diastolic filling, and that verapamil improves filling in the majority of pts.

Effects of nifedipine on LV funtion: To assess the effects of nifedipine on exercise-induced LV dysfunction in pts with CAD, 11 pts were studied this year by radionuclide cineangiography during placebo, nifedipine, propranolol, and combined nifedipine plus propranolol therapy. Neither nifedipine, propranolol, nor nifedipine plus propranolol altered mean ejection fraction for the group of patients at rest, although ejection fraction at rest decreased more than 5% in 3 pts during nifedipine and in one additional patient during nifedipine plus propranolol treatment. With exercise, ejection fraction was unchanged by propranolol, but increased in response to nifedipine and to the combination of nifedipine plus propranolol. Hence, the change in ejection fraction from rest to exercise, an index of reversible LV ischemia, was favorably influenced by nifedipine and nifedipine plus propranolol. Abnormal regional wall motion with exercise, present in all pts during control studies, improved in 6 pts during nifedipine therapy and in 7 during nifedipine plus propranolol, but in zero pts during propranolol. Thus, nifedipine and nifedipine plus propranolol improved LV function during exercise in many pts with CAD. However, since nifedipine and the combination of nifedipine plus propranolol may depress LV function at rest in certain pts, cautious monitoring of pts is warranted during administration of these agents.

Relative myocardial and vascular effects of verapamil, nifedipine and diltiazem: There are currently three calcium blocking agents being used clinically. Indirect evidence suggested that the relative vasodilator and myocardial depressant potencies of these drugs may differ. Last year we compared the relative effects of verapamil, diltiazem and nifedipine on hemodynamics and LV function during partial coronary occlusion in dogs without evidence of LV decompensation. We found that verapamil produced the greatest negative inotropic effect; however, it also manifested the most pronounced beneficial effect on LV diastolic filling. We hypothesized that the differences observed

among these three agents will have important clinical implications. One issue raised by this study was whether either or both of these drugs is dangerous to use in CHF. This year, therefore, we extended our observations, comparing the relative effects of verapamil and nifedipine during acute partial coronary occlusion in dogs with LV filling pressures elevated to 27 mmHg, (i.e., dogs with LV decompensation). Our results demonstrated that when the drugs were administered at doses causing a decrease in arterial pressure of 10%, verapamil further increased left atrial pressure (+61%), while nifedipine led to a diminution (-18%). Moreover, while verapamil reduced both global (-6%) and ischemic zone (-6%) ejection fraction, ejection fraction was increased by nifedipine both globally (+21%) and regionally in the ischemic zone (+22%). These findings suggest that verapamil may be more dangerous than nifedipine when administered to pts with LV decompensation and ischemia.

Contrasting effects of verapamil and nifedipine on pH of ischemic myocardium: During acute coronary occlusion (CO) verapamil decreases and nifedipine increases contractile function of the ischemic zone. However, it is not known whether either response influences ischemic injury. Thus, we measured intramyocardial pH with special fiber optic probes in open chest dogs, which were pretreated with either verapamil or nifedipine to reduce mean aortic pressure by 10 mmHg. pH was measured during drug treatment for 15 min after total LAD CO at depths of 3 mm (measuring epicardial pH) and 6 mm (measuring endocardial pH). There was less depression in pH in the ischemic zones during verapamil compared to either nifedipine or placebo therapy. Hence, verapamil but not nifedipine ameliorates ischemic acidosis during coronary occlusion. The pH differences were not explained by differences in myocardial blood flow or mean arterial pressure, and were only partly explained by changes in heart rate. Thus, the mechanism responsible for improved pH in the ischemic zones produced by verapamil probably involves a direct effect on ischemic myocardium, and may relate to the decrease in ischemic zone contractile function caused by verapamil, but not nifedipine.

ALTERATIONS IN CORONARY VASOCONSTRICTOR TONE AS A CAUSE OF ANGINA PECTORIS

Traditionally, angina pectoris has been considered to result from a fixed stenosis limiting flow to the myocardium. As myocardial oxygen demands increase in response to any stress, the stenosis limits the augmentation in flow that normally occurs, causing myocardial oxygen demands to exceed myocardial oxygen supply: ischemia and ultimately anginal pain result. The recognition of vasospastic angina (Prinzmetal or variant angina) focused attention on the possibility that coronary vessels could manifest alterations in intrinsic tone. However, the concept of vasospastic angina was limited, being conventionally understood as angina occurring at rest that is precipitated by a profound decrease in coronary flow which results from spasm-induced total or near total occlusion of a coronary artery. We have hypothesized that a spectrum of vasoconstrictor tone may exist, with Prinzmetal angina representing only one extreme of the spectrum. We are thus examining the hypothesis that increases in vasoconstrictor tone occur that are insufficient to cause a reduction in rest myocardial flow, but severe enough to compromise the capacity of the coronary artery to augment its flow in response to stress. Implicit in this hypothesis is the concept that dynamic, spontaneous alterations in the degree of coronary vasoconstriction occur; angina would thereby appear at variable thresholds of exercise stress, depending upon the degree of vasoconstrictor tone.

Angina caused by abnormal coronary arteriolar vasoconstriction in pts with atypical angina: To study the mechanism of angina pectoris in pts with atypical angina, great cardiac vein flow and arterio-venous (AV) lactates were measured at rest and with atrial pacing (to achieve heart rates between 130 and 150), followed by coronary angiography. These measurements were obtained under control conditions and following two different vasoconstrictor stimuli: immersion of the pt's hand in ice water and during infusion of ergonovine. The stress of pacing ordinarily increases coronary flow and decreases coronary vascular resistance. Pts experiencing angina during pacing demonstrated less of an increase in LAD flow and less of a decrease in LAD resistance than pts who did not experience angina both under control conditions and during administration of vasoconstrictor stimuli. The pts experiencing angina also demonstrated abnormal changes in AV lactate extraction, suggesting that the chest pain was indeed due to myocardial ischemia. The abnormal lactate metabolism was exacerbated when pacing was performed during either vasoconstrictor stimulus.

These findings indicate that some pts with atypical angina exhibit inappropriately high vasoconstrictor tone that can be exacerbated or produced by vasoconstrictor stimuli. This abnormality can lead to reduced vasodilator reserve of coronary arteries, thereby either causing angina or lowering the threshold at which it occurs. Coronary angiography performed during cold-pressor or ergonovine testing failed to demonstrate signifiant epicardial coronary artery narrowing, indicating that the locus of increased resistance involves the small coronary vessels, most likely at the arteriolar level.

Most pts who experienced angina pectoris during pacing under control conditions experienced angina at a lower paced heart rate under the influence of either cold-pressor testing or ergonovine infusion. This suggests myocardial ischemia occurred at a lower threshold of myocardial oxygen consumption during coronary vasoconstrictor stimuli, a finding compatible with the concept that the precipitation of angina was facilitated by actual vasoconstriction. That active vasoconstriction did occur was more definitively indicated by the finding that when angina was precipitated by pacing during administration of either cold-pressor stimulation or ergonovine infusion, coronary flow actually decreased in several patients despite the fact that MVO_2 increased (as indicated by the product of heart rate and arterial pressure).

Another conclusion with important diagnostic implications derived from these studies relates to the utility of traditional ergonovine testing to determine the presence or absence of vasospasm as a potential cause of chest pain. The standard procedure to determine the role of coronary arterial spasm as a cause of angina is to administer an ergonovine challenge. Absence of large vessel constriction, determined angiographically, is taken as evidence that spasm is not responsible for symptoms. Our results demonstrate that reliance on the large vessel "spasm" response to ergonovine as an indication of coronary vasoconstriction can cause diagnostic errors, as angina may be due to vasoconstriction of coronary arterioles, which cannot be visualized at arteriography. Studies are now in progress in which we are evaluating the clinical significance of these findings and are assessing the influence of vasodilator therapy in controlling the symptoms of patients presenting with such clinical and hemodyamic abnormalities.

Natural history of patients with coronary artery disease who have no or mild symptoms: By 1975 it was known that coronary bypass operation relieved symptoms in pts with CAD and enhanced survival in pts with stenosis of the left main coronay artery. Operation was therefore offered to pts with symptoms refractory to medical management, and to pts with left main disease. What was not known, however, was whether operation enhanced survival in pts whose symptoms were well controlled and who did not have left main disease. Our pt population was not large enough to undertake a randomized trial comparing operatively vs. medically treated pts with CAD and no or mild symptoms. However, we decided a study determining whether subgroups of such pts could be identified who were at high or at low risk of dying could provide considerable insight as to the indications for operation in pts whose symptoms were well controlled medically. The study was initiated in 1975 and this year a major portion of that study was completed.

We followed from 6 to 67 mos (average, 25 mos) 147 asymptomatic or mildly symptomatic pts with CAD, who did not have significant left main coronary occlusion and had ejection fractions >20%. Significant 1 vessel disease (VD) was present in 28% of pts, 2 VD in 31% and 3 VD in 41%. During follow-up there have been 8 deaths, yielding a mortality of 3% per year for the entire group: mortality was 1.5% per year for 1 and 2 VD, but 6% per year for 3 VD. Better definition of high and low risk subgroups of pts with 3 VD was accomplished by exercise testing. Despite a history of mild symptoms, 25% of pts with 3 VD exhibited poor exercise capacity after discontinuation of beta blockers and nitrates: of these, 40% either died (20%) or developed progressive symptoms requiring operation (20%). The resulting annual mortality was 9%. Of pts with good exercise capacity, only 22% either died (7%) or developed progressive symptoms (15%); annual mortality was 4%. Thus, prognosis is excellent in pts with no or mild symptoms who have 1 or 2 VD. Pts with 3 VD who have good exercise capacity documented by objective testing have an annual mortality of 4% per year. Because pts with 3 VD and poor exercise capacity have an extremely grave prognosis, it would appear reasonable to recommend coronary bypass operation for this subgroup, even in the absence of supporting data derived from a definitive randomized study. The choice between medical and operative treatment of those pts with 3 VD and good exercise capacity is more ambiguous; therefore definitive recommendations cannot be unequivocally established until additional long-term survival data of both medically and operatively treated pts are available.

Balloon dilatation of the coronary arteries (PTCA): Over 200 pts have undergone PTCA at the NIH. This past year we determined the incidence, clinical presentation and management of restenosis following originally successful PTCA. Restenosis occurred in 20% of pts who had an initially successful procedure. All but 1 of 18 pts with restenosis had recurrence of typical angina as an indication of restenosis, and symptoms recurred 2 weeks to 4 months following initial PTCA. When symptoms recurred after restenosis they could either be mild or severe; one of the 18 pts presented with an acute myocardial infarction. Other than 2 pts developing total occlusion, all pts have undergone repeat PTCA and in all it was successful. Restenosis following the second procedure occurred in 3 pts (19%), a restenosis rate similar to that observed following the initial procedure. The remainder of the pts are asymptomatic. Thus, we conclude that although restenosis can occur in pts undergoing PTCA, a second procedure can be performed safely and effectively so that a total of 92% of pts with an initially successful result are ultimately improved.

NHLBI Type II Coronary Intervention Study: The primary aim of this randomized double-blind prospective study is to determine whether lowering cholesterol with cholestyramine and diet in pts with premature CAD and Type II hypercholesterolemia will retard the progression of CAD. The major criterion we are employing to answer this question is whether there is regression of anatomic disease or evidence of slower progression, conclusions that will be based on coronary angiograms obtained at initiation into study and after 5 years of treatment. By October, 1981, we had: 1) concluded data collection for the program; 2) discharged all pts into other programs of NIH or to their source of private care; 3) transferred all basic data to the coordinating center. Analysis of data is currently under way and it is anticipated the primary analysis will be completed and conclusions as to the results of the study determined by October, 1982.

HYPERTROPHIC CARDIOMYOPATHY

Characterization of hypertrophy in pts with HCM: HCM is a disease of cardiac muscle characterized by a hypertrophied, nondilated LV. During the last 2 years we have analyzed and defined the morphologic distribution of the hypertrophy by 2-dimensional (2-D) echo in a large group of pts. In these studies we found several patterns of distribution of LV hypertrophy, ranging from localized to widespread. Because extent and distribution of hypertrophy 1) influences symptomatic status, 2) determines ease of diagnosing the disease, and 3) may relate to propensity to sudden death, we have continued to expand our 2-dimensional echocardiographic analyses.

Patterns of inheritance in HCM: HCM is a genetically transmitted disease in some families. However, the frequency with which HCM is familial, the precise mode of inheritance and the genetic influences (if any) on morphologic distribution of hypertrophy are largely unresolved issues. To determine patterns of inheritance of HCM, nearly 300 pts from 70 families were studied by M-mode and 2-D echo. Inspection of pedigrees showed the disease was familial with autosomal dominant pattern in 56% of the index cases, but was sporadic in 44%. Probands commonly showed symptoms (81%), subaortic obstruction (53%), and diffuse and marked LV hypertrophy (51%). In contrast, affected relatives were usually asymptomatic (72%), without obstruction (94%), and had less diffuse LV hypertrophy (60%). Pts with the familial or sporadic forms did not differ in phenotypic expression of HCM. In the genetically transmitted form of HCM, 2-D echo findings demonstrated that morphologic expression of distribution of hypertrophy in closely related pts is variable, and usually is markedly dissimilar. Hence, in HCM 1) genetic transmission is common, usually with an autosomal dominant pattern; 2) sporadic occurrence is present in a substantial proportion of families; 3) a variety of phenotypic expressions occur which may have genetic as well as nongenetic etiologies.

Other 2-D echocardiographic studies of pts with HCM: HCM may or may not be associated with subaortic obstruction. Obstruction results from systolic anterior motion of the mitral valve (SAM) into the LV outflow tract. However, the determinants of SAM are not completely understood. We defined the role of LV outflow tract orifice size in determining subaortic obstruction. We found that the presence or absence of obstruction correlated with LV outflow tract area: pts with smaller areas had the greatest likelihood of having obstruction. Using 2-D echocardiography we also discovered a newly recognized cause of dynamic subaortic obstruction. Obstruction usually occurs in the presence of a small LV outflow tract when the anterior mitral leaflet moves forward in systole and comes into apposition with the ventricular septum. However, using both

M-mode and 2-D echocardiography we found a group of pts in which only the posterior mitral leaflet moved anteriorly in systole and approached or contacted the septum. Most of these individuals demonstrated obstruction at catheterization. Pathologic observations in 3 pts who died showed abnormal lengthening of only the middle scallop of the posterior mitral leaflet. This elongated segment appears to come into apposition with the septum bypassing between the caudal attachments of the anterior mitral leaflet. Septal myotomy-myectomy performed in 2 pts markedly diminished SAM of the posterior mitral leaflet.

Changes in the magnitude of LV outflow obstruction with time and associated clinical alterations: Although spontaneous regression or appearance of LV outflow obstruction has been known to occur, the significance of this hemodynamic alteration has not been established. We evaluated the clinical course of 23 pts with HCM who showed substantial spontaneous reduction or increase in magnitude of subaortic obstruction. The period of time elapsing between the two evaluations ranged from 1 to 21 years. Of 8 pts with reduction or loss of obstruction, 6 (75%) deteriorated clinically and 2 (25%) were stable. Of the 15 pts with increased gradient, 10 (66%) deteriorated clinically and 5 (34%) were unchanged. Atrial fibrillation appeared responsible for increased symptoms in 3 pts with loss of obstruction and 1 pt with increased gradient. Thus, 1) substantial changes in magnitude of obstruction occur in some pts with HCM as part of the natural history of the disease; 2) such hemodynamic alterations are usually associated with progression of the disease and clinical deterioration.

VALVULAR HEART DISEASE

Determining Optimal Time for Operating on Pts with Aortic Regurgitation (AR)

When a pt with AR develops significant symptoms, operation is clearly indicated. However, the optimal time to operate on a pt with significant AR but without symptoms was unclear. Over the past several years we have undertaken a series of studies in which we demonstrated that once an asymptomatic pt with AR develops left ventricular dysfunction, (as detected by echocardiography, radionuclide angiography, or contrast angiography), significant symptoms develop in the large majority of pts within 2-3 years. Moreover, when symptomatic pts with LV dysfunction are operated upon, approximately 60% die within 3-4 years of operation from refractory LV decompensation: prognosis is considerably worse in symptomatic patients with poor treadmill exercise capacity than in those with good exercise capacity. Hence, our studies were instrumental in establishing a strategy for following and evaluating pts with AR: asymptomatic pts with good LV function should be followed until evidence of LV dysfunction develops; at that time, operation should be performed. On the other hand, if a pt presents with AR and also has LV dysfunction, operation should not be delayed. Over the past year we have continued our studies in an attempt to develop additional insights about this clinically important problem.

The natural history of asymptomatic pts with AR and normal LV function: The fear of a pt with AR developing irreversible LV failure has led to the recommendation that operation be performed in all pts with significant AR, even if LV function is normal. In such pts, however, the natural history without operation is unknown. We therefore performed serial studies in 79 asymptomatic pts with normal LV ejection fraction (EF) determined by radionuclide angiography at rest. During mean follow-up of 47 months (range 6 to 110 months) no pt died and 12 have undergone aortic valve replacement: 11 developed symptoms and 1 developed LV dysfunction without symptoms. By life table analysis, the percent of pts who did

not require operation was 90% at 3 years, 81% at 5 years and 75% at 7 years. In the 12 operative pts there were no operative or late deaths, LV EF increased from 45% pre-op to 57% postop, and echo LV diastolic dimension decreased from 73 mm pre-op to 53 mm postop. Thus in asymptomatic pts with normal LV function death is rare and less than 4%/yr require op because symptoms of LV dysfunction develop. When operation is delayed until symptoms or LV dysfunction develop, postop survival is excellent, and LV size and function improve. Hence, "prophylactic" operation to preserve LV function should not be performed in asymptomatic pts with severe AR and normal LV function.

Relation between duration of pre-operative LV dysfunction and postoperative reversal of dysfunction: Although we previously demonstrated that preoperative LV function is an important predictor of postop prognosis in pts with AR, the relation between the duration of preop LV dysfunction on reversibility of dysfunction is unknown. We therefore studied 30 pts with subnormal (<29%) preop echo LV fractional shortening (FS) undergoing op. In 9 pts LV dysfunction was documented 18-57 months preop (prolonged); in 7 pts LV dysfunction developed less than 12 months preop (brief); in 14 pts duration of preop LV dysfunction was unknown. Postoperative LV diastolic dimension fell to a lower level in pts with brief LV dysfunction than in pts with prolonged LV dysfunction (52 vs. 59 mm, $p<.02$), and postop EF was higher, (65 vs. 43%, $p<.005$). Postop EF in pts with brief LV dysfunction was also higher than in pts with unknown duration of dysfunction (48%, $p<.01$). Thus in pts with AR and LV dysfunction, the duration of preop dysfunction is an important determinant of the postop reversibility of dysfunction. These data indicate that pts with AR should undergo op without delay once LV dysfunction is demonstrated.

Utility of LV ejection fraction during exercise in determining operation in asymptomatic pts: LVEF at rest has proven of prognostic value in pts with AR. Since abnormal LVEF during exercise precedes LV dysfunction at rest, many physicians have recommended operation in asymptomatic pts with AR once abnormalities of LVEF during exercise are detected. To assess the prognostic implications of the exercise LVEF, we studied 70 consecutive asymptomatic pts with serial radionuclide angiograms. Although 21% of pts with a fall in EF from rest to exercise developed symptoms (mean follow-up 22 months), 79% remained asymptomatic (mean follow-up 28 months). The 11 pts who developed symptoms underwent op. Exercise LVEF failed to improve more than 5% in only those 3 pts with subnormal preop rest EF. Thus, 1) LVEF decreases during exercise early in the course of AR, 2) neither the initial exercise EF nor change in EF from rest to exercise predicts which pts will develop symptoms or deterioration in resting LVEF over a 2 year period, 3) depressed preop exercise LVEF in symptomatic pts improves postop as long as resting EF is normal. These data indicate that exercise LVEF has unproven prognostic value and should not yet be used alone to determine timing of operation in asymptomatic AR pts.

COLLABORATIVE STUDIES

The Cardiology Branch has collaborated in a large number of studies with other Institutes. One area of continued interest has been the effects on cardiac function of agents successfully employed in the chemotherapy of cancer.

Prospective evaluation of adriamycin cardiotoxicity by radionuclide cineangiography: Clinical LV dysfunction develops in pts who receive adriamycin therapy, but the frequency with which adriamycin produces depression of LV function is unknown. We therefore studied 25 consecutive pts by radionuclide

angiography before and after adriamycin therapy (mean total dose 521 mg/m^2, range 480-555 mg/m^2). All pts had normal resting LVEF before adriamycin. Adriamycin produced clinical LV dysfunction in only 4 pts (17%). However, the drug depressed resting LV function in 76% and impaired LV function during exercise in another 20%. Hence, adriamycin depressed LV function in 96% of pts. Nonetheless, these data indicate that in the absence of preceding heart disease, it is uncommon for adriamycin to cause clinically significant LV dysfunction.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01661-07 CB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Natural History of Asymptomatic and Mildly Symptomatic Patients with Coronary Artery Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Kenneth M. Kent	Head, Cardiovascular Diagnosis	CB	NHLBI
Other:	Douglas R. Rosing	Senior Investigator	CB	NHLBI
	Carolyn J. Ewels	Biologist	CB	NHLBI
	Lewis C. Lipson	Senior Investigator	CB	NHLBI
	Robert O. Bonow	Senior Investigator	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI_ NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.7	.2	.5

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To determine the prognosis of patients with coronary artery disease who have no symptoms or mild symptoms, such patients have been followed prospectively. Patients with single and double vessel disease have had an excellent prognosis. However, prognosis of patients with three vessel disease has been poor. Patients with three vessel disease who manifest poor exercise capacity have an annual mortality of 9%; those who manifest a good exercise capacity have an annual mortality of 4%. This contrasts to an annual mortality of only 1.5% in those patients with single or double vessel disease.

PHS-6040
(Rev. 2-81)

Project Description:

Coronary artery bypass operations are being recommended to many patients with double or triple vessel coronary artery disease, including those with either mild or no symptoms, in order to enhance survival. Such an approach appears justified when the survival curves of patients following coronary bypass operation are compared to the previously published survival curves of non-operated patients. However, the survival curves of these previous natural history studies are derived from the results of patients evaluated in large referral centers and followed in the 1960's and early 1970's. Thus it is important to determine whether these survival statistics of non-operated coronary patients, commonly compared to those of operated patients, pertain to asymptomatic or mildly symptomatic patients evaluated and treated in the mid and late 1970's.

One hundred forty seven asymptomatic or mildly symptomatic patients with coronary artery disease, who did not have significant left main coronary occlusion and had ejection fractions >20%, have been followed prospectively from 6 to 67 months (avg. 25 months). Significant obstruction of one coronary artery was present in 28% of patients, two coronary arteries in 31%, and three coronary arteries in 41%. Ejection fraction was $\geq$ 55% in 69% of patients. During follow-up there have been eight deaths, yielding a mortality of 3% per year for the entire group: mortality was 1.5% per year for single and double vessel disease, but 6% per year for triple vessel disease. Better definition of high and low risk subgroups of patients with three vessel disease was accomplished by exercise testing. Despite a history of mild symptoms, 25% of the patients with triple vessel disease exhibited poor exercise capacity on exercise testing after discontinuation of beta blockers and nitrates: of these, 40% either died (20%) or developed progressive symptoms requiring operation (20%) yielding an annual mortality of 9%. Of the patients with good exercise capacity, only 22% either died (7%) or developed progressive symptoms (15%), with an annual mortality of 4%.

In summary, the results of our study demonstrate that in asymptomatic or mildly symptomatic patients with single or double vessel coronary artery disease, 1) the event rate of sudden death or of the development of severe irreversible left ventricular dysfunction is low, and 2) most patients who deteriorate symptomatically can be operated upon successfully. Thus, it appears reasonable to manage these patients medically, with operation deferred until symptoms develop that compromise the patient's lifestyle. Mildly symptomatic patients with triple vessel disease, however, do not have such a favorable outlook. Although those patients with good exercise capacity documented by objective testing have an annual mortality rate of 4% per year, annual mortality of those patients with three vessel disease and poor exercise capacity is 9%, and an additional 4% per year develop progressive symptoms requiring operation. Hence, the prognosis of this latter group is grave enough to warrant operative intervention, even in the absence of a definitive randomized study. The choice between medical and operative treatment of those patients with triple vessel disease and good exercise capacity is more ambiguous; therefore, definitive recommendations cannot be unequivocally established until additional long-term survival data of both medically and operatively treated patients are available.

Publications: Kent, KM., Rosing, DR., Ewels, CJ., Lipson, L., Bonow, R, Epstein, S.E. Prognosis of asymptomatic or mildly symptomatic patients with coronary artery disease. Am J Cardiol 49: 1923-1931, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01670-06 CB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
The Natural History of Aortic Regurgitation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Douglas R. Rosing	Head, Cardiovascular Diagnosis	CB	NHLBI
Other:	Robert O. Bonow	Senior Investigator	CB	NHLBI
	Richard O. Cannon	Clinical Associate	CB	NHLBI
	Rita M. Watson	Clinical Associate	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI,NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.2	.1	.1

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

One hundred five patients with varying degrees of aortic regurgitation in either NYHA Functional Class I or II have been evaluated and are being followed prospectively in order to assess the natural history of this disease. Admission studies included echocardiography, rest and exercise radionuclide angiography, measurement of pulmonary artery wedge pressure during exercise, and 24-hour ambulatory monitoring. These studies should allow us to identify more sensitive indicators of impending clinical deterioration than are presently available.

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PHS-6040
(Rev. 2-81)

Project description:

In patients with aortic regurgitation (AR), we presently employ the development of symptoms as the primary indication for proceeding with valve replacement. However, the results of operation are often less than optimal in terms of symptom relief and long-term survival. In order to develop more sensitive indicators of deterioration of cardiac function in asymptomatic patients with AR, extensive clinical and hemodynamic evaluations are being performed. To date, 105 functional class I or II patients with aortic regurgitation have been evaluated with echocardiograms, graded treadmill exercise tests, radionuclide cineangiograms at rest and during supine exercise, measurement of pulmonary artery wedge pressure response to intense supine exercise and 24-hour ambulatory monitoring. The average follow-up time has been 44 months. Since less than 20 patients were processed in the past year, the data has not been updated and the following numbers reflect only the first 93 patients entered into the study. This group has been divided into 21 asymptomatic patients with mild aortic regurgitation, 59 asymptomatic patients with moderate to severe AR, and 13 patients who have undergone aortic valve replacement after having been initially followed in the Natural History Study. These latter 13 were operated on either because of decreasing left ventricular function or the development of symptoms while under observation. Only one patient from this group has died at this point, and he had mild AR; significant coronary artery disease was found at the time of post-mortem examination.

In comparison to this group, the same intensive evaluation has been carried out on 52 patients who were referred either with moderate to severe aortic regurgitation and significant symptomatology or evidence of left ventricular dysfunction. These patients have had their aortic valves replaced and are being re-evaluated six months after operation. There have been seven deaths in this group. Four were at the time of operation and two occurred within the first month of discharge. Both of these latter patients had also undergone aortic root replacement for aortic root disease.

Since the study has been initiated, we have already revised our indications for valve replacement. This change in policy has resulted not only from the data obtained in the present study, but also from data obtained on patients evaluated prior to initiations of this study. Valve replacement is performed not only in patients with significant symptomatology, but also when left ventricular systolic function is compromised, as evidenced by echocardiography (left ventricular systolic dimension greater than 55 mm).

Exercise radionuclide angiography and exercise-induced changes in pulmonary capillary wedge-pressure also are measurements capable of distinguishing the three groups of mildly symptomatic or asymptomatic patients listed above. Since only 13 patients have crossed over from the medical to surgical groups, we have not as yet been able to establish specific criteria applicable to these tests which will be helpful in predicting clinical or hemodynamic deterioration. However, it is clear that hemodynamic measurements made during intense exercise are not sensitive indicators of left ventricular dysfunction and these measurements are no longer being made.

Significant high grade ventricular ectopy has been detected in this group, but its significance is uncertain. With 24 hr ambulatory monitoring, 36/80 pts with AR with no or mild symptoms had Lown class 4A or 4B ventricular ectopy while on no antiarrhythmic treatment. Ventricular ectopy was not related to severity of AR or presence of coronary disease (CAD) but did correlate with the status of left ventricular function asessed by radionuclide angiography.

In 35/47 additional consecutive pts studied prior to aortic valve replacement, high grade ventricular ectopy was also found. Ventricular tachycardia was present in 18 non-operative patients and 21 operative pts (NS). Rx was initiated in only 4 non-operative pts with ventricular tachycardia. Non-operative pts without ventricular tachycardia were not placed on Rx and high grade ventricular ectopy continued in 22/31 pts who underwent repeat ambulatory monitoring. Six months after AVR, ambulatory monitoring was repeated in 35 pts and 32 continued to have high grade ventricular ectopy. Only one non-operative pt died (ventricular tachycardia, CAD). Four pts died at the time of aortic valve replacement (2=high grade ventricular ectopy, 1 = CAD, high grade ventricular ectopy) and two died within the first month after aortic valve replacement. Only one other pt has died after aortic valve replacement with a mean follow-up time of 29 mo. These data indicate that high grade ventricular ectopy is common in mildly or severely symptomatic pts with AR and occurs independently of the presence of CAD. However, high grade ventricular ectopy has not been associated with increased mortality during follow up of either non-operative or operative patients.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01672-06 CB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Maintenance of a Computerized Clinical Data Bank for Cardiology Patients

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Douglas R. Rosing	Head, Cardiovascular Diagnosis	CB	NHLBI
Other:	Charles McIntosh	Senior Surgeon	CB	NHLBI
	Gerald D. Stoner	Head, Applied System Program Section	DMB	DCRT
	Gail Greenberg	Computer Technician	CB	NHLBI
	Lavonne Dragt	Computer Technician	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)
Surgery Branch, NHLBI

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	.1	.9

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A computerized clinical data bank has been established for all cardiology patients. Inpatient and outpatient data will include symptom description, and X-ray, ECG, catheterization, echocardiogram, exercise stress test, ambulatory monitor and radionuclide angiogram results.

114

PHS-6040
(Rev. 2-81)

Project description: A computerized data bank has been established for cardiology patients in order to provide easy access to patient data for clinical and investigative purposes. Included in the data base is information from both outpatient and inpatient visits as well as the identification of procedures and diagnoses generated at other institutions. The data includes symptom description, X-ray, ECG, catheterization, radionuclide, exercise stress test, ambulatory monitor and echocardiogram interpretations, listing of current medications, and disposition of the patient. In addition, with the assistance of the "MIS" system, a printout of the catheterization results is generated for the medical records. Data accumulation was begun on January 1, 1978 and current and retroactive information has been entered on all of our patients. In the winter of 1979, the data base of the Cardiology Branch and the one already in service under the direction of Dr. Charles McIntosh of the NHLBI Surgical Branch were merged into one system, thus facilitating access to data on all patients with cardiovascular disease. Queries of the data base for clinical and research purposes have averaged about 20 per month. A summary of available "pertinent" clinical data is now obtained for each outpatient visit to the Cardiology and Surgery Clinics in order to make chart reviews easier at these visits.

At the present time, there are 6,584 patients entered into the data base. Of these patients, approximately 4,000 have undergone some cardiac operation. Over the past year, the opportunity to perform more complex statistical analysis (STAT program) has become available to those querying the system. This upgrading has increased the number of queries requested, and there are several manuscripts in process based mainly on data obtained from the data bank.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01750-04 CB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Verapamil: A New Approach to the Treatment of Hypertrophic Cardiomyopathy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Douglas R. Rosing	Head, Cardiovascular Diagnosis	CB	NHLBI
Other:	Barry J. Maron	Senior Investigator	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Behtesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.5	.4	.1

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Verapamil has been administered to over 200 patients with hypertrophic cardiomyopathy in order to try to improve their quality of life. Approximately 60% have remained on the medication for at least six months (range 6-57). These patients have manifested an improvement in subjective symptomatic status as well as exercise capacity over this period of time.

116

PHS-6040
(Rev. 2-81)

Project description: The primary approach to symptomatic therapy in patients with hypertrophic cardiomyopathy (HCM) is the use of beta blocking agents. We previously demonstrated that verapamil reduces left ventricular outflow tract obstruction and improves exercise capacity and subjective symptomatology in hospital in such patients. To assess the chronic effects of verapamil in HCM, over 200 patients whose lifestyle was unacceptable despite propranolol therapy were begun on oral verapamil in hospital between September, 1977 and January, 1982. Less than 10% of patients were not discharged on verapamil due to: side effects (1 death), non-compliance, and symptomatic deterioration. The major reason the drug was stopped in patients discharged on verapamil was because symptoms were unrelieved or recurred. Less than 2% of patients discharged on verapamil had the drug discontinued because of non-physiologic drug side effects. Six patients have died while on chronic drug treatment. Approximately 60% of patients discharged on the drug have remained on verapamil (6-57 mos). Adverse hemodynamic effects experienced in the patients included seven deaths, ten episodes of pulmonary congestion, three of hypotension, two cases of sinus arrest, approximately 10% incidence of development of junctional rhythm or Wenkebach 2 heart block. Non-cardiovascular side effects include upper abdominal discomfort in two patients, frequent constipation, and occasional descriptions of hair loss. In almost all cases of hypotension, junctional rhythm, 2 heart block, and non-cardiovascular problems, the drug was continued at reduced dosage, although therapeutic efficacy was sometimes compromised because of an inability to use higher doses. Of the patients started on verapamil in hospital, approximately 45% have either improved their functional class by at least 1 grade and/or have increased their exercise duration by 15%. Due to the ongoing work in this area, we have been able to identify those subgroups of patients who appear to be at increased risk for developing complications secondary to verapamil administration, and who should not receive the medication. Thus, verapamil continues to offer a much needed new therapeutic approach to the treatment of HCM.

Publications:

Rosing, D.R., Condit, J.R., Maron, B.J., Kent, K.M.,Leon, M.B., Bonow, R.O., Lipson, L.C., Epstein, S.E.: Verapamil therapy. A new approach to the pharmacologic treatment of hypertrophic cardiomyopathy. III. Effects of long-term administration. Am. J. Cardiol. 48: 545-553, 1981.

Rosing, D.R., Verapamil - therapeutic success, but how? Internat. J. Cardiology. 1:253-255, 1982.

Rosing, D.R., Epstein, S.E., Verapamil in the treatment of hypertrophic cardiomyopathy. Annals Int. Med. 96: 670-672, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01761-04 CB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Percutaneous Transluminal Coronary Angioplasty

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Kenneth M. Kent	Head, Cardiovascular Diagnosis	CB	NHLBI
Other:	Douglas R. Rosing	Senior Investigator	CB	NHLBI
	Carolyn J. Ewels	Biologist	CB	NHLBI
	Lewis C. Lipson	Senior Investigator	CB	NHLBI
	Robert O. Bonow	Senior Investigator	CB	NHLBI
	Stephen L. Bachrach	Physicist	NM	CC
	Michael V. Green	Chief, Applied Physics Sec.	NM	CC
	Charles L. McIntosh	Senior Surgeon	SB	NHLBI
	Michael Jones	Senior Surgeon	SB	NHLBI

COOPERATING UNITS (if any)
Department of Nuclear Medicine
Surgery Branch

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.5	.3	.2

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Non-operative treatment with a balloon catheter to reduce coronary arterial stenoses is being evaluated. Dilatation of the coronary narrowing improves the patients symptoms, objective assessment of exercise capacity and coronary blood flow during exercise.

PHS-6040
(Rev. 2-81)

Project Description:

101 patients have undergone percutaneous transluminal coronary angioplasty (PTCA). All patients had symptoms of angina pectoris prior to the procedure; fourteen patients were mildly symptomatic, 67 had moderate symptoms (angina with usual activities) and 20 patients had severe symptoms including symptoms at rest. Eighty one patients had single vessel coronary artery disease, fifteen had double vessel disease, and in 5 patients PTCA was attempted in a bypass vein graft. Sixty five (65%) patients had initial angiographic improvement in the coronary stenosis. In the remaining 36 patients the arterial stenosis could either not be crossed with the balloon catheter (22 patients), was not compressable (10 patients) when the balloon was inflated to its maximum distending pressure, or inflation of the balloon resulted in arterial dissection and abrupt occlusion of the artery necessitating urgent coronary bypass operation (4 patients). Of the 65 patients in whom there was initial angiographic improvement of the arterial stenosis, 52 were both initially improved and have remained symptomatically stable up to 18 months after the procedure, and 13 developed recurrent severe symptoms with angiographically documented restenosis at the site of the original angioplasty. PTCA was repeated in eleven of the 13 patients. Seven of the eleven patients remain stable after the second PTCA. All cases of restenosis occurred within the first three months after PTCA. Of the stable patients, 27 have returned for coronary arteriograms six months after the initial PTCA, ten have shown a reduction in the degree of stenosis compared to the immediate post-PTCA calculated stenosis (further improvement), 12 remain angiographically stable and five had had <20% increase in the degree of stenosis.

To determine whether the improvement in the angiographic appearance of the coronary arteries would reduce exercise induced abnormalities in myocardial perfusion and function, perfusion (TL 201) and gated blood pool (Tc99m) scans at rest and exercise were performed prior to, 2 to 21 days after, and six months following PTCA. Before PTCA 55 of the 65 patients who ultimately had a successful angiographic result were stable enough to undergo exercise testing off medication. During exercise 51/55 patients developed angina and 50 of the 55 patients developed left ventricular wall motion abnormalities (ventricular tachycardia terminated exercise in one patient). Left ventricular ejection fraction either remained unchanged or fell in all. PTCA reduced the average stenosis from 84% to 30% and the average gradient across the stenosis was reduced from 63 mm Hg to 20 mm Hg ($p<.01$). Following PTCA, none of these patients developed angina during exercise. Left ventricular function and perfusion were unchanged at rest. During exercise, only three of the 50 patients with prior wall motion abnormalities developed such abnormalities after PTCA. Ejection fraction during exercise increased in each of the patients from the rest value, and was greater than the value during exercise prior to PTCA, in all except three. Average ejection fraction during exercise was 55% $\pm$ 3% pre-PTCA and increased to 66% $\pm$ post-PTCA ($p<.01$). In all patients who remained symptomatically stable, myocardial function at rest and during exercise was stable at the six months study following PTCA. Thus, the results demonstrated that angiographic improvement obtained with PTCA leads to improved exercise capacity, LV function, and LV perfusion, all presumably reflecting augmentation of functionally important coronary blood flow.

Five major complications of the procedure have occurred. One patient in whom the procedure was unsuccessful who developed an acute myocardial infarction 12 hours after PTCA. Myocardial infarction was uncomplicated. Three patients had an apparently uneventful angioplasty procedure but post procedure angiography demonstrated arterial (intimal) dissection at the site of the stenosis. Over 15 to 60 minutes, blood flow through the arteries decreased, symptoms and electrocardiographic changes documented myocardial ischemia. In a fourth patient intimal dissection proximal to the stenosis occurred resulting in arterial occlusion. All four patients underwent urgent coronary revascularization operations. No deaths have occurred in the series.

We conclude PTCA can be performed in selected patients with coronary artery disease. Initial angiographic improvement can be obtained in two-thirds of the patients undergoing the procedure. At this point, the complication rate of the procedure appears to be low. Finally, when angiographic improvement occurs, it is accompanied by restoration of functionally important myocardial blood flow during exercise.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 HL 01792-03 CB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Ventricular Septal Contour in Hypertrophic Cardiomyopathy: Two-dimensional Echo Analysis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Barry J. Maron	Senior Investigator	CB	NHLBI
Other:	Timothy P. Blair	Guest Worker	CB	NHLBI
	John S. Gottdiener	Guest Worker	CB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.7	.6	.1

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Two-dimensional echocardiography permits non-invasive assessment of ventricular septal contour. A variety of septal contours are present in patients with hypertrophic cardiomyopathy which are highly specific markers for that disease.

121

PHS-6040
(Rev. 2-81)

Project description: Hypertrophic cardiomyopathy is a disease of cardiac muscle in which the ventricular septum is usually involved prominently in the cardiomyopathic process. Because ventricular septum contour may have implications regarding diagnosis and advisability of operation, 70 patients with hypertrophic cardiomyopathy and 57 patients with other heart diseases or normal hearts were studied by two-dimensional echo using primarily parasternal long axis and apical views. A variety of contours were identified, with the most common (25 patients, 35%) being "lemon-shaped" (convex to both left ventricle and right ventricle). Ventricular septum contour in other patients were convex toward left ventricle and flat (30%) or concave (25%) to right ventricle, or flat toward left ventricle and convex to right ventricle (10%). In contrast, in each control patient the right and left septal surfaces were parallel. Patients with hypertrophic cardiomyopathy also showed variable distribution of ventricular septum hypertrophy. In most patients (59, 84%) hypertrophy involved all of ventricular septum and was maximal in the middle 1/3. Of note, in 8 (12%) of patients hypertrophy was discretely confined to the cephalad 1/3 of ventricular septum resembling a "tumor nodule", and in 3 (4%) patients was present only in apical regions. Hence: in patients with hypertrophic cardiomyopathy 1) a wide variety of septal contours are present that represent highly specific echo markers for that disease; 2) defining the distribution ventricular septum hypertrophic may be useful in the planning for septal myotomy and myectomy.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01798-02 CB

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Clinical and Morphologic Features of Patients with Hypertrophic Cardiomyopathy and Ventricular Septal Hypertrophy Localized to the Apical Regions of the Left Ventricle (Apical Hypertrophic Cardiomyopathy")

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Barry J. Maron	Senior Investigator	CB, NHLBI
Other:	Robert O. Bonow	Senior Investigator	CB, NHLBI
	William C. Roberts	Chief, Pathology Branch	PB, NHLBI
	Stephen E. Epstein	Head, Cardiology Branch	CB, NHLBI

COOPERATING UNITS (if any)

Pathology Branch, NHLBI

LAB/BRANCH

Cardiology Branch

SECTION

Clinical Physiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.11	.1	.01

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Patients with hypertrophic cardiomyopathy may have hypertrophy confined to the apex of the left ventricle. Identification of this form of the disease can not be made by conventional M-mode echocardiography but requires two-dimensional echocardiography.

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PHS-6040
(Rev. 2-81)

Project Description:

The clinical and morphologic features of a unique subgroup of patients with hypertophic cardiomyopathy is described. In each of these patients, ventricular septal hypertrophy was shown by two-dimensional echocardiograpy or necropsy examination to be virtually confined to the apical portion of the left ventricular wall. M-mode echo is unreliable in making the diagnosis of hypertrophic cardiomyopathy since the conventional path of the M-mode beam does not traverse the areas of left ventricular hypertrophic cardiomyopathy, as evidenced by genetic transmision of hypertrophic cardiomyopathy, as evidenced by genetic transmission of hypertrophic cardiomyopathy with other patterns of left ventricular hypertrophy identified in family members of these patients and marked disorganization of cardiac muscle cells was present in the left ventricular wall of one patient who died suddenly and was studied at necropsy.

The apical distribution of left ventricular hypertrophy was, in the study patients, often associated with: 1) T wave inversion (although not "giant T wave inversion"); 2) "hour-glass" angiographic appearance of left ventricle in systole with mid-cavity oblitration and a small, poorly contractile distal apical segment of left ventricle; 3) mid-ventricular systolic pressure difference between the small distal cavity and larger proximal chamber.

Of note, our patients with apical distribution of hypertrophy differ in several important respects from nonobstructive "apical hypertrophic cardiomyopathy" described in Japanese patients. This observation suggests that genetic and racial differences may account for the different clinical and morphologic features of this form of hypertrophic cardiomyopathy in the United States and Japan.

Publications:

1. Maron, B.J., Bonow, R.O., Roberts, W.C., Epstein, S.E. Clinical and Morphologic features of patients with hypertrophic cardiomyopathy and ventricular septal hypertrophy localized to the apical regions of the left ventricle ("apical Hypertrophic Cardiomyopathy"). Amer J Cardiol 49:1838-1848, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01799-02 CB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Sudden Death in Hypertrophic Cardiomyopathy: Clinical Profile in 78 patients

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Barry J. Maron	Senior Investigator	CB, NHLBI
Other:	William C. Roberts	Chief, Pathology Branch	PB, NHLBI
	Stephen E. Epstein	Head, Cardiology Branch	CB, NHLBI

COOPERATING UNITS (if any)

Pathology Branch, NHLBI

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.11	.1	.01

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Patients with hypertrophic cardiomyopathy who die suddenly stress are most commonly young and asymptomatic. The majority were performing sedentary or mild exertion at the time of death. Hemodynamic findings, ventricular septal thickness and ECT alterations do not appear to predict which patients are at highest risk for sudden death.

PHS-6040
(Rev. 2-81)

Project Description:

The clinical profile of 78 patients with hypertrophic cardiomyopathy who died suddenly (or experienced cardiac arrest and survived) was analyzed. At the time of sudden death or cardiac arrest the majority of patients were young (70% were < 30 years of age), without functional limitation (54%) and performing sedentary or minimal physical activity (61%). Nineteen (24%) of the 78 patients were taking propranolol in apparently adequate dosages, inidcating that this drug does not convey absolute protection against the occurrence of sudden death.

No clinical or morphologic parameter analyzed proved particularly reliable in identifying patients at risk for sudden death. While the vast majority of patients who died suddenly had a markedly increased ventricular septal thickness of > 20 mm (48 of 64 patients, 75%), mean septal thickness was similar in patients who died suddenly (25.2 $\pm$ 0.9 mm) and in control patients hypertrophic cardiomyopathy who have survived (23.6 $\pm$ 0.8 mm). An abnormal electrocardiogram was present as commonly in those patients who have survived, i.e., 51 of 53, 96%). In addition, no obstruction or left ventricular end-diastolic pressure, proved to be characteristic of the patients with hypertrophic cardiomyopathy cardiomyopathy who died suddenly.

Publications: Maron, B.J., Roberts, W.C., Epstein, S.E. Sudden death in Hypertrophic Cardiomyopathy: Clinical Profile in 78 patients. Circulation 65:1388-1394, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 04002-02 CB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Comparative effects of verapamil and nifedipine on exercise capacity and symptoms in patients with hypertrophic cardiomyopathy.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. R. Rosing	Senior Investigator	CB, NHLBI
Other:	R. O. Cannon	Clinical Associate	CB, NHLBI
	R. O. Bonow	Senior Investigator	CB, NHLBI
	R. M Watson	Clinical Associate	CB, NHLBI
	S. E. Epstein	Chief, Cardiology Branch	CB, NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.3	.2	.1

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Verapamil, a calcium channel blocking agent, has been shown to be an effective new therapeutic agent for the treatment of patients with hypertrophic cardiomyopathy. In order to determine whether other drugs of this type will be equally effective, nifedipine was compared with verapamil and placebo administration in a double-blind randomized study. Results indicate that both verapamil and nifedipine can improve exercise capacity and symptomatic status in patients with hypertrophic cardiomyopathy, but patients must be carefully chosen because serious adverse drug effects can occur. Although verapamil seems to be more effective than nifedipine in the treatment of this disorder, nifedipine may be helpful in patients who do not tolerate or respond to verapamil.

PHS-6040
(Rev. 2-81)

Project Description:

Verapamil (V) increases exercise capacity (EC) and improves subjective symptomatology (SS) relative to placebo in patients with hypertrophic cardiomyopathy (HCM). To assess whether nifedipine (N) has similar beneficial properties, EC and SS was evaluated in 27 patients with HCM during oral V, N and P administration. Drugs were given in a randomized double-blind fashion in low and high dosages. Maximal EC, independent of dosage, was determined for each drug in each patient. On P, average maximal EC was 5.5 $\pm$ 3.3 min. On V it was 7.5 $\pm$ 3.3 min ($p = 0.02$); on N, 6.5 $\pm$ 2.9 min ($p = 0.11$). Heart rates at rest and at maximal EC were similar for P and N, but significantly reduced wth V. Thirteen patients described their symptomatic status as "best" on V, 7 did the same for P and 6 for N. Only 4 stated they felt "worse" on V, compared to 7 for N and 12 for P. The results of exercise and synmptmatic evaluation agreed 75% of the time. Four patients were excluded from the study due to drug side effects: V = hypotension (1), N = syncope (1), pulmonary edema (1), P = hypotension (1). These findings indicate that V is more effective than N in improving exercise capacity and subjective sumptomatic status in patients with HCM. N may be helpful, however, in some patients who do not tolerate or respond to V.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 04010-02 CB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Clinical Efficacy of Nifedipine With and Without Propranolol in Patients with Exertional Angina Pectoris due to Coronary Artery Disease.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. R. Rosing	Senior Investigator	CB, NHLBI
Other:	R. O. Cannon	Clinical Associate	CB, NHLBI
	R. M Watson	Clinical Associate	CB, NHLBI
	R. O. Bonow	Senior Investigator	CB, NHLBI
	S. E. Epstein	Chief, Cardiology Branch	CB, NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.2	.1	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

An in-hospital study has been initiated evaluating the effectiveness of nifedipine therapy alone or in combination with propranolol in patients with chronic stable angina pectoris. Fourteen patients completed the protocol. The combination of nifedipine and propranolol resulted in an improvement in exercise capacity. All drugs were well tolerated for the most part.

PHS-6040
(Rev. 2-81)

Project Description:

During placebo administration, mean exercise duration was 5.2 ± 1.7 minutes with 13/14 patients stopped by angina and other by leg pain. During propranolol administration (80-320 mg/day), mean exercise time was 5.5 ± 2 minutes with 13/14 patients again stopped by angina and the other by dyspnea and leg fatigue. On nifedipine (12 patients - 120 mg/day, one - 80 mg/day, one 40 mg/day), exercise duration was 5.6 ± 2.6 minutes with all patients having angina as their end point. On the combination of the nifedipine (same dose as above) and propranolol (maximum dose 160 mg/day), exercise duration was 6.1 ± 2.9 minutes with 10/14 patients stopped by angina and the other four with leg fatigue. Applying analysis of variance to these results demonstrated p values > .2 for all comparisons except that between the use of the combination of drugs and placebo where p = 0.06.

These results do not agree with a number of other investigations describing nifedipine, propranolol and the combination effective antianginal therapy. The reason for this disparity is not clear, but is presently being evaluated through further data analysis of drug blood levels and the results of radionuclide studies at rest and with exercise.

The only adverse effects experienced was that one patient developed postural hypotension and sinus tachycardia on doses of nifedipine above 40 mg/day, and another patient became very fatigued on doses of propranolol above 80 mg/day.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 04015-02 CB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Early Degradation of Collagen after Acute Myocardial Infarction
(Revised Title)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Richard O. Cannon, III	Clinical Associate,	CB, NHLBI
Other:	Edith Speir	Technician	CB, NHLBI
	Jagdish Butaney	Research Associate	PB, NHLBI
	Victor J. Ferrrans	Chief, Ultra Structure Section,	PB, NHLBI

COOPERATING UNITS (if any)
Ultra Structure Section, Pathology Branch

LAB/BRANCH
Cardiology Branch

SECTION
Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.1	.1	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cardiac rupture and aneurysm formation following acute myocardial infarction may be related to degradation of structurally important collagen molecules by inflammatory cell proteolytic enzymes. Using a rat model of acute myocardial infarction, significant collagen degradation was found to occur in the first twenty-four hours following experimental infarction. This degradation was associated with a marked increase in proteolysis in the infarct region. This collagen degradation was inhibited by eliminating inflammatory cells by prior whole body irradiation. Electron mircroscopic studies demonstrated collagen breakdown in control infarcts and preservation in infarcts of irradiated, leukopenic animals confirming an important role of white cell proteases in early degradation of collagen after acute myocardial infarction.

/31

PHS-6040
(Rev. 2-81)

Project Description:

Following acute myocardial infarction, proteolysis of necrotic tissue occurs in association with a massive influx of white cells. To determine whether collagen is also degraded (which might decrease wall strength) and, if so, whether white cell proteases are implicated, the amount of collagen was measured in tissues from border zone (BZ), ischemic zone (IZ) and normal zone (NZ) from 24-hour old infarcts produced in normal rats and rats made leukopenic (WBC < 300/mm^3) by whole body irradiation. In addition, the numbers of polymorphonuclear leukocytes (PMN's) in the BZ were counted in tissue sections from other similarly treated animals. Precipitation of tissue homogenates with trichloroacetic acid was used to separate partially hydrolyzed from large, presumably intact collagen molecules. The latter were measured as hydroxyproline (nmol/mg dry wt. of tissue; mean ± SE).

	Control (n = 9)	Leukopenic (n = 23)
NZ	9.0 ± 1.0	11.5 ± 1.5
BZ	3.7 ± 0.7*	9.3 ± 1.1
IZ	4.8 ± 0.9*	9.3 ± 1.1

(* = $p < 0.005$ vs control NZ)

The numbers of PMN's /mm^2 of BZ were: control (n = 5) 416 ± 43, leukopenic (n = 5) 75 ± 11 ($p = < 0.001$). Tissue sections from BZ and IZ for electron microscopy revealed collagen breakdown in control rats, as opposed to a greater degree of collagen preservation in leukopenic rats. Thus, 1) at 24 hours after experimental acute myocardial infarction there is significant degradation of collagen, especially in the border zone where the concentration of PMN's is highest; 2) degradation occurs to a much lesser extent in leukopenic rats, indicating an important role of white cell proteases in early degradation of collagen after acute myocardial infarction.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 04022-02 CB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Hemodynamic Effects of Nifedipine in Patients with Hypertrophic Cardiomyopathy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. R. Rosing	Head, Cardiovascular Diagnosis	CB, NHLBI
Other:	R. O. Cannon	Clinical Associate	CB, NHLBI
	R. M Watson	Clinical Associate	CB, NHLBI
	R. O. Bonow	Senior Investigator	CB, NHLBI
	H.G. Ostrow	Electronics Engineer	CB, NHLBI
	S. E. Epstein	Chief, Cardiology Branch	CB, NHLBI

COOPERATING UNITS (if any)

Department of Computer Research and Technology

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.1	.05	.05

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Verapamil, a calcium channel blocking agent, has been shown to have beneficial hemodynamic effects in patients with hypertrophic cardiomyopathy. To assess whether other drugs of this type have similar effects, nifedipine was administered buccally in the Catheterization Laboratory to patients with this disorder. Although nifedipine appeared to decrease left ventricular outflow obstruction, its lack of effect on left ventricular systolic pressure and early diastolic filling raises questions as to whether it will be as effective as verapamil.

PHS-6040
(Rev. 2-81)

Project Description:

Verapamil (V) lowers LV outflow gradients (GRAD) and improves diastolic filling in patients with hypertrophic cardiomyopathy (HCM). To assess whether nifedipine (N) has similar beneficial effects, N was given to 37 HCM pts at catheterization. Heart rate (HR), systolic blood pressure (SBP), mean pulmonary wedge pressure (PCW), cardiac index (CI), LV end diastolic volume (LVDV) and LV GRAD were measured before (C) and after a total of 10-30 mg of buccal N (* = $p < 0.005$).

	HR	SBP	PCW	CI	GRAD	LVDV
C	78 ± 13	126 ± 15	15 ± 3	2.9 ± 0.8	56 ± 37	100%
N	91 ± 13*	103 ± 13*	17 ± 9	3.5 ± 0.8*	65 ± 43	131 ± 33%

Although LV systolic pressure did not decrease with N administration, the lack of change in LV GRAD when CI increased indicates a reduction in LV outflow obstruction which appears to be secondary to increased LVDV. Prior readionuclide studies with V have shown improved diastolic filling rate (PFR) and decreased time to peak filling Rate (TPFR). Although TPFR was decreased by N, PFR was unchanged. Thus although N increases CI in pts with HCM, its lack of effect on LV systolic pressure raises questions as to whether it will be as effective as V in symptomatic control of pts with HCM.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 04024-02 CB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Side Effects of Oral Verapamil Therapy in Patients with Cardiac Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Douglas R. Rosing	Head, Cardiovascular Diagnosis	CB NHLBI
Other:	Terry Rehder	Pharm. D.	Pharm. CC
	Stephen E. Epstein	Chief, Cardiology Branch	CB NHLBI

COOPERATING UNITS (if any)
Department of Pharmacy

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.1	.05	.05

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Verapamil administration has resulted in occasional life threatening side effects related to its physiological actions. A number of less serious complaints have also been elicited in patients, but these "adverse effects" have rarely either limited the dosage of the drug which could be administered or prevented its continued use. A protocol was developed in order to determine the frequency, significance, and relationship to verapamil administration of these "adverse effects". The incidence of side effects is also being determined for propranolol, a beta adrenergic blocking agent, another cardiac medication used in these same patient groups. Early results indicate that the drug appears to be well tolerated, but a few unsuspected potential side effects may have been uncovered.

Project Description: Verapamil has been characterized as a well tolerated, safe drug in the cardiologic literature. Most of the reports regarding the type and incidence of side effects from this drug have originated from studies performed outside the United States. During our initial experience with verapamil, we became aware of the potential life threatening adverse effects which could occur as a result of the physiological effects of verapamil--negative inotropy, peripheral vasodilatation, conduction system depression. Less obvious was the capacity of this agent to produce less serious side effects.

In order to determine the frequency and significance of non-life threatening side effects, a patient questionaire was designed. At the present time, 42 patients on chronic verapamil therapy have been interviewed. When it became apparent that the list of possible side effects would be legion, the questionaire was also administered to patients on beta adrenergic blocking drugs for comparison. Eventually, patients on no cardiac drugs will also be interviewed. The demographic data obtained include:

			Disease		Drug	
Sex		Age (X)	CAD	HCM	Verapamil	Propranolol
Women	15	49 Y/O	2	13	14	1 (CAD)
Men	35	50 Y/O	16	19	28	7 (IHCM)

Side effects of the two medicines are compared in the attached table. The major side effects definitely related to verapamil administration appear to be constipation, arthralgias, and weight gain. In patients on propranolol, frequent adverse effects included depression, memory loss, worsening of temperament, decreased libido, fatigue, and urinary urgency. Only five of over 20 patients started on verapamil have been unable to tolerate the drug. The reasons for discontinuing its use were upper gastrointestinal discomfort, rash, nausea and vomiting (2) and memory loss.

In summary, although minor side effects are common verapamil appears to be a well-tolerated drug and few patients have had to discontinue its use or modify their dosage because of non-life threatening drug side effects.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 04025-01 CB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Transmural pH Gradient in Ischemia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Rita M. Watson	Clinical Associate	CB	NHLBI
Other:	David A. Markle	Engineer	DRS	BEIB, MES
	Douglas A. McGuire	Technician	DRS	BEIB, MES
	John I. Peterson	Engineer	DRS	BEIB, CES
	Seth R. Goldstein	Chief, MES	DRS	BEIB, MES
	Randolph E. Patterson	Senior Investigator	CB	NHLBI

COOPERATING UNITS (if any)
Biomedical Engineering and Instrumentation Branch

LAB/BRANCH
Cardiology Branch

SECTION
Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The subendocardium is more vulnerable to ischemia than is the epicardium. Transmural gradients in intramyocardial pressure, oxygen tension, myocardial blood flow and glycolytic enzyme activities have been documented. Although ischemia causes acidosis, the existence of a transmural gradient of pH has not been documented or quantitated. By means of specially designed fiberoptic pH probes implanted in the ischemic and normal canine myocardium, we documented the existence of a large transmural pH gradient over 3-4 mm. The close spatial proximity of regions with markedly different pH undoubtedly contributes to physiological abnormalities and arrhythmogenesis in ischemic hearts.

PHS-6040
(Rev. 2-81)

Project Description:

The subendocardium (Endo) is more vulnerable to ischemia than is the epicardium (Epi). Although ischemia causes acidosis, the existence of a transmural gradient of pH (TMG-pH) has not been documented or quantitated. We effected a critical LAD stenosis in 4 open chest dogs. Simultaneous pH data were recorded via specially designed fiberoptic pH probes implanted in the ischemic zone (IZ) and normal zone (NZ) Endo (depth 5.5-8 mm) and Epi (depth 3-4 mm). Coronary flow and perfusion pressure (CPP) were decreased by partial occlusion (PO) to abolish reactive hyperemia and held constant. Microspheres confirmed reduced blood flow and endo/epi flow ratio. Grouped data are shown for mean CPP (mmHg) and pH:

	Mean CPP	IZ Endo	IZ Epi	NZ Endo
Control	108	7.45	7.42	7.49
10 min PO	49	7.22	7.41	7.47
30 min PO	49	6.95	7.31	7.44

Pacing increased the IZ "endo" pH from pH 6.55 to pH 7.20 as the probe migrated from Endo toward Epi. We conclude: 1) There is a large TMG-pH over 3-4 mm in the IZ. 2) that very gradient may cause erroneous conclusions regarding interventions in myocardial ischemia unless pH probe position is stabilized, and 3) the close spatial proximity of regions with markedly different pH must contribute to physiological abnormalities in ischemic hearts.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 04026-01 CB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Contrasting Effects of Verapamil and Nifedipine on pH of Ischemic Myocardium

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Y.M. Ro	Guest Worker	CB
Other:	R.E. Patterson	Senior Investigator	CB
	D. Markle	Engineer	MES, BEIB, DRS
	S. Goldstein	Chief, MES	MES, BEIB, DRS
	R. Aamodt	Chief, Whole Body Counter Section	NM, CC
	S.E. Epstein	Chief, Cardiology Branch	CB, NHLBI

COOPERATING UNITS (if any)

DRS, BEIB, Mechanical Engineering Section
Department of Nuclear Medicine, Clinical Center

LAB/BRANCH
Cardiology Branch

SECTION
Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	2.0	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We tested the effects of Verapamil and Nifedipine on intramyocardial pH during myocardial ischemia to determine whether Verapamil and Nifedipine improve myocardial acidosis following coronary occlusion in dogs. Verapamil, but not Nifedipine, improved acidosis of ischemic myocardium. There was no difference among groups treated with Verapamil, Nifedipine or placebo in ischemic zone myocardial blood flow or mean aortic pressure. Verapamil, but not Nifedipine, lowered heart rate. However, analysis of covariance showed higher ischemic zone pH for any heart rate during Verapamil compared to Nifedipine or placebo, indicating direct effect of Verapamil independent of heart rate. Thus, the mechanism of improved ischemic zone pH by Verapamil probably involves a direct effect on ischemic myocardium, e.g., decreased ischemic zone contractile function.

PHS-6040
(Rev. 2-81)

Project Description:

During acute coronary occlusion (CO) verapamil (V) decreases and nifedipine (N) increases contractile function of the ischemic zone (IZ). However, it is not known if either response influences ischemic injury. Thus, we measured intramyocardial pH with special fiber optic probes in 20 open chest dogs. We pretreated 9 dogs with V and 9 with N to reduce mean aortic pressure (MAP) by 10 mmHg; 11 dogs received placebo (P). pH was measured during drug treatment for 15 min after total LAD CO at depths of 3 mm (Ep) and 6 mm (EN). Results at 5 min follow for pH-IZ:

Group	pH-Ep	pH-EN	MBF-IZ	HR	MAP	LAP
P	6.90	6.79	0.18	174	103	6.0
N	6.88	6.75	0.14	163	87θ	6.2
V	7.09*+	6.96*+	0.22	132*+	92+	7.8

*p <.05, V vs P; +p <.05, V vs N, θp <.05 N vs P, by analysis of covariance (COV) for 5, 10, and 15 min post CO. MBF = myocardial blood flow. COV showed higher pH-IZ for any HR during V compared to N or P (p <.02), indicating a direct effect of V independent of HR. Hence, V - but not N - ameliorates ischemic acidosis curing CO. The pH differences are not explained by differences in MBF or MAP and are only partly explained by changes in HR. Thus, the mechanism of improved pH-IZ by V probably involves a direct effect on ischemic myocardium, e.g., decreased IZ contractile function.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 04027-01 CB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Abnormal coronary artery vasoconstriction in patients with angina pectoris and insignificant fixed coronary artery disease.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Richard O. Cannon, III	Clinical Associate,	NHLBI, CB
Other:	Rita M. Watson	Clinical Associate,	NHLBI, CB
	Douglas R. Rosing	Head, Cardiovascular Diagnosis	NHLBI, CB
	Stephen E. Epstein	Chief, Cardiology Branch	NHLBI, CB

COOPERATING UNITS (if any)

None

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.5	.4	.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Patients with exertional chest pain are often found to have insignificant coronary artery disease or normal coronary arteries at coronary angiography. Coronary artery spasm of large epicardial vessels is freguently suggested as an etiology for anigina in these patients, though standard challenges such as cold pressor testing and ergonovine generally fail to provide angiographically demonstrable spasm. Thirteen patients with chest pain and insignificant coronary artery disease underwent the same vasoconstrictor challenges along with rapid atrial pacing. Patients who experienced chest pain during the testing demonstrated biochemical evidence of myocardial ischemia, and coronary blood flow measurement revealed a significantly lower drop in coronary vascular resistance compared to patients who did not experience chest pain. No patient demonstrated large vessel coronary artery spasm. These studies suggest that coronary arterioles may have inadequate vasodialtor reserve in some patients resulting in angina pectoris under certain conditions.

PHS-6040
(Rev. 2-81)

Project Description:

In order to study the mechanism of angina pectoris (AP) in patients with insignificant fixed coronary artery (CA) disease, 13 patients with insignificant LAD CA disease underwent great cardiac vein flow (GCV-F), resistance (GCV-R) and AV lactate determinations at rest and with atrial pacing to HR 130 - 150, followed by coronary angiography. Values represent final paced HR as percent of resting control, mean ± SE.

	ΔGCV-F	ΔGCV-R	ΔAV lactate
No AP (n = 6)	↑ 67 ± 34	↓ 46 ± 4	↑ 8 ± 22
AP (n = 7)	↑ 58 ± 12	↓ 20 ± 7*	↓ 32 ± 24

(* = < 0.025 vs NO AP GCV-R)

The patient's hand was then immersed in ice water (CPT) for 30 seconds, followed by submaximal and maximal pacing. Five of the 7 patients who experienced AP during the control study experienced AP at a lower paced HR with CPT. Values respresent final paced HR as percent of resting control.

	ΔGCV-F	ΔGCV-R	ΔAV Lactate
No AP (n = 5)	↑ 92 ± 30	↓ 38 ± 13	↑172 ± 53
AP (n = 8)	↑ 45 ± 8*	↓ 11 ± 4**	↓ 20 ± 11**

(* = p < 0.05, ** = p > 0.025 vs respective NO AP). Coronary angiography performed during repeat CPT demonstrated insignificant epicardial CA narrowing.

Ergonovine (E) .15 mg IV was then given after repeat control measurements: 2/12 pts experienced AP (GCV-F ↓ 13%, ↓ 21% and GCV-R ↑ 41% respectively, from control). No significant changes occurred in the 10 pts without AP. Still under the influence of E, pacing to 130 - 150 was instituted in these 10 pts; 5 experienced AP. Values represent precent change from resting control.

	ΔGCV-F	ΔGCV-R	ΔAV Lactate
No AP (n = S)	↑ 61 ± 16	↓ 31 ± 5	↑ 49 ± 48
AP (n = S)	↑ 47 ± 5	↓ 12 ± 1*	↓ 49 ± 21**

(* = p < 0.005, ** = p < 1.05 vs respective No AP)

Coronary angiography during E demonstrated no significant CA luminal narrowing or focal spasm.

These studies suggest some patients with AP and insignificant fixed CA disease may have abnormal vasodilator reserve of resistance vessels which may be unmasked or exacerbated by vasoconstrictor stimuli such as CPT and E.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 04028-01 CB

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Reversibility of Left Ventricular Dysfunction in Patients with Aortic Regurgitation.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Robert O. Bonow	Senior Investigator,	CB, NHLBI
Douglas R. Rosing	Senior Investigator,	CB, NHLBI
Barry J. Maron	Senior Investigator,	CB, NHLBI
Michael Jones	Senior Investigator,	SB, NHLBI
Charles L McIntosh	Acting Chief,	SB, NHLBI
Stephen E Epstein	Chief, Cardiology Branch	CB, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Cardiology Branch

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Preoperative left ventricular (LV) function is an important determinant of postop prognosis in patients with aortic regengitation (AR). To determine the influence of duration of LV dysfunction on reversibility of LV dysfunction, we studied 30 patients with LV dysfunction (subnormal echo fractional shortening) and preserved exercise capacity before and after aortic valve replacement. Patients with brief LV dysfunction (< 12 months) preop manifested greater reduction in postop LV diastolic dimension by echocardiography and greater increase in postop LV ejection fraction by radionuclide angiography than patients with prolonged LV dysfunction (> 18 months). Thus, in patients with AR and LV dysfunction, duration of preop LV dysfunction is an important determinant of postop reversibility of LV dysfunction. These data indicate that patients with AR should undergo valve replacement without delay once LV dysfunction is demonstrated.

PHS-6040
(Rev. 2-81)

Project Description: Preoperative left ventricular (LV) function is an important predictor of postop prognosis in patients with aortic reguigitation (AR). Previous studies have shown that LV dysfunction is reversible after operation to a greater extent in patients with good exercise capacity compared to patients with impaired exercise capacity. Not all patients with good exercise capacity, however, demonstrated improved LV function after operation. To determine the influence of duration of preop LV dysfunction on revsersibility of LV dysfunction , we studied 30 patients with subnormal (< 29%) preop echo LV fractional shortening (FS) undergoing aortic valve replacement: 23 patients underwent operation because of onset of symptoms (sx) and 7 patients were asymptomatic (asx). All patients had good preop exercise capacity. In 9 patients (6 sx, 3 asx) LV dysfunction was documented 18 - 57 months preop (prolonged); in 7 patients (6 sx, 1 asx) LV dysfunction developed less than 12 months preop (brief); in 14 patients (11 sx, 3 asx) duration of preop LV dysfunction was unknown. Both preop echo LV dimensions and FS and radionuclide angiographic ejection fraction (EF) were not different between patients with brief vs. prolonged LV dysfunction. However, postop LV diastolic dimension fell to lower levels in patients with brief LV dysfunction than with prolonged LV dysfunction (52 $\pm$ 2 vs. 59 $\pm$ 7 mm, $p < .02$) and postop EF was higher (65 $\pm$ 6 vs. 43 $\pm$ 15%, $p < .005$). Postop EF in patients with brief LV dysfunction was also higher than in patients with unknown duration of LV dysfunction (48 $\pm$ 11%, $p < .01$). Thus, in patients with AR and LV dysfunction the duration of preop LV dysfunction is an important determinant of the postop reversibility of LV dysfunction. These data indicate that patients with AR should undergo aortic valve replacement without delay once LV dysfunction is demonstrated.

Publications: none

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 04029-01 CB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
The Natural History of Asymptomatic Patients with Aortic Regurgitation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Robert O. Bonow	Senior Investigator,	CB, NHLBI
Douglas R. Rosing	Senior Investigator,	CB, NHLBI
Stephen L. Bacharach	Physicist,	APS, NM, CC
Michael V Green	Chief,	APS, NM, CC
Stephen E Epstein	Chief,	CB, NHLBI

COOPERATING UNITS (if any)

Applied Physics Section, Nuclear Medicine Dept. CC, NIH

LAB/BRANCH
Cardiology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.6	.6	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Left ventricular (LV) function is an important determinant of postoperative prognosis in patients with aortic regurgitation . Hence, to preserve LV function, aortic valve replacement has been recommended to many asymptomatic patients with normal LV function. However, the natural history of such patients is unknown. We therefore assessed the natural history of 79 asymptomatic patients with normal LV ejection fraction by radionuclide angiography. During mean follow up of 47 months, 11 patients underwent operation because of symptoms and a twelfth because of asymptomatic LV dysfunction. By life table analysis, 75% ± 8% of patients did not require operation at 7 years. There were no deaths. In the operative patients, LV function improved postop. Since death is rare, less than 4%/year require operation because of symptoms or LV dysfunction, and LV function improves after operation, "prophylactic" valve replacement to preserve LV function is not required in asymptomatic patients with AR and normal LV function.

145

Project Description: Two thirds of asymptomatic patients with aortic regurgitation (AR) and left ventricular (LV) dysfunction develop symptoms requiring operation within 2 to 3 years. It is therefore reasonable to recommend aortic valve replacement in asymptomatic patients with LV dysfunction. In asymptomatic patients with severe AR and normal LV function, aortic valve replacement has been recommended to preserve LV function. In such patients, however, the natural history without operation is unknown. We therefore performed serial studies in 79 asymptomatic patients with normal radionuclide angiographic LV ejection fraction (EF) at rest: 64 patients had 3 to 4+ AR on aortic root cine, and the other 15 patients had pulse pressure > 70 mm Hg. During mean follow-up of 47 mos (range 6- 110 mos) no patients died and 12 patients have undergone aortic valve replacement: 11 developed symptoms and one developed LV dysfunction without symptoms. By life table analysis, the percent of patients who did not require operation was 90 ± 4% at 3 yrs, 81 ± 6% at 5 yrs, and 75 ± 8% at 7 yrs. In the 12 operative patients, there were no operative or late deaths (postop follow-up 9 - 66 mos, mean 39 mos), LVEF increased (45 ± 6% pre, 57 ± 9% postop; $p < .001$), and echo LV diastolic dimension decreased (73 ± 4 mm pre, 53 ± 4 mm postop $p < .001$). Thus, in asymptomatic patients with normal LV function, death is rare, and less than 4%/year require aortic valve replacement because symptoms or LV dysfunction develop. When aortic valve replacement is delayed until symptoms or LV dysfunction develop, postop survival is excellent, and LV size and function improve postop. Hence, "prophylactic" aortic valve replacement to preserve LV function should not be performed in asymptomatic patients with severe AR and normal LV function.

Publications: none

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 04030-01 CB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Nifedipine and Propranolol Effects on Cardiac Function During Exercise in Coronary Artery Disease.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Robert O. Bonow	Senior Investigator,	CB, NHLBI
Douglas R. Rosing	Senior Investigator,	CB, NHLBI
Richard O. Cannon	Medical Staff Fellow	CB, NHLBI
Rita M. Watson	Medical Staff Fellow	CB, NHLBI
Lewis C. Lipson	Senior Investigator,	CB, NHLBI
Sebastian T. Palmeri	Expert Consultant	CB, NHLBI
Kenneth M. Kent	Senior Investigator,	CB, NHLBI
Stephen L. Bacharach	Physicist,	APS, NM, CC
Michael V. Green	Chief,	APS, NM, CC
Stephen E. Epstein	Chief,	CB, NHLBI

COOPERATING UNITS (if any)
Applied Physics Section, Nuclear Medicine Dept. CC, NIH

LAB/BRANCH
Cardiology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The effects of nifedipine and propranolol on exercise left ventricular (LV) function were assessed by radionuclide angiography in 11 patients with coronary artery disease and chronic stable angina pectoris. Propranolol did not alter regional or global LV function during exercise. However, both nifedipine and nifiedipine + propanolol increased exercise LV ejection fraction and improved exercise regional wall motion abnormalities compared to placebo and propanolol. Hence, nifiedipine reduces evidence of exercise-induced LV dysfunction in many patients with coronary artery disease.

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PHS-6040
(Rev. 2-81)

To assess the effects of nifedipine (N) and propranolol (P) on exercise-induced left ventricular (LV) dysfunction in patients with coronary artery disease (CAD) and chronic stable angina, we studied 11 patients by radionuclide angiography during placebo (C), nifedipine (40 - 120 mg/day), propranolol (80 - 320 mg/day), and combined nifedipine + propranolol. During placebo, LV ejection fraction (EF) at rest was normal in 10 pts. Neither nifedipine, propranolol, nor nifedipine + propranolol altered mean EF at rest (C = 53 $\pm$ 9%, N = 52 $\pm$ 8%, P 53 $\pm$ 9%, N+P = 54 $\pm$ 8%), although EF at rest decreased > 5% in 3 patients during nifedipine and in one additional patient during nifedipine + propranolol. With exercise, propranolol did not affect EF (C = 50 $\pm$ 11%, P = 48 $\pm$ 10%; NS) but nifedipine increased EF (56 $\pm$ 13%) compared to placebo and propranolol (both $p < .05$). Nifedipine + propranolol also increased EF during exercise (54 $\pm$ 11%) compared to placebo ($p < .02$) and propranolol ($p < .01$) but was unchanged from nifedipine alone. Hence, the change in EF from rest to exercise, an index of reversible LV ischemia, was favorably ($p < .05$) influenced by nifedipine and nifedipine + propranolol compared to placebo and propranolol. Abnormal regional wall motion with exercise, present in all patients during placebo, improved in 6 patients during nifedipine, in 7 patients during nifedipine + propranolol but in 0 patients during propranolol. Thus, nifedipine and nifedipine + propranolol improve LV function during exercise in many patients with CAD and chronic stable angina. However, since nifedipine and nifedipine + propranolol may depress LV function at rest in selected patients, cautious monitoring of patients is warranted during administration of these agents.

Publication: none

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 04031-01 CB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Serial Left Ventricular Function During Exercise in Aortic Regurgitation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Robert O. Bonow	Senior Investigator,	CB, NHLBI
Douglas R. Rosing	Senior Investigator,	CB, NHLBI
Kenneth M. Kent	Senior Investigator,	CB, NHLBI
Lewis C. Lipson	Senior Investigator,	CB, NHLBI
Stephen L. Bacharach	Physicist,	APS, NM, CC
Michael V. Green	Chief,	APS, NM, CC
Stephen E. Epstein	Chief,	CB, NHLBI

COOPERATING UNITS (if any)

Applied Physics Section, Nuclear Medicine Dept. CC, NIH

LAB/BRANCH
Cardiology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.6	.6	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Left ventricular (LV) ejection fraction at rest is an important determinant of long-term prognosis in patients with aortic regurgitation. To assess the prognortic implications of the LV ejection fraction during exercise, we studied 70 initially asymptomatic patients by serial radionuclide angiography, of whom 11 developed symptoms requiring operation during follow-up. These data indicate that 1) LV ejection fraction decreases during exercise compared to the value at rest early in the course of aortic regurgitation, including 90% of patients with echo LV systolic dimension > 40 mm; 2) initial exercise ejection fraction does not predict either development of symptoms nor deterioration in rest ejection fraction; and 3) depressed preop exercise ejection fraction in symptomatic patients improves postop. Hence, the exercise ejection fraction has unproven prognostic vaue and should not be used alone in determining timing of operation in asymptomatic patients.

149

PHS-6040
(Rev. 2-81)

Left ventricular (LV) ejection fraction (EF) at rest has proven of prognostic value in patients with aortic regurgitation (AR). Abnormal LVEF during exercise (Ex) precedes LV dysfunction at rest in many patients. To assess the prognostic implications of the ex LVEF, we studied 70 consecutive asymptomatic patients with serial radionuclide angiograms. Initially, 18 patients had an increase in EF from rest to ex (+ΔEF) and 52 had -ΔEF, including 47 of 52 patients (90%) with echo LV systolic dimensions (SD) > 40 mm. No patient with +ΔEF developed symptoms during follow-up. Although 11 patients with -ΔEF (21%) developed symptoms (mean follow-up 22 mo), 41 patients (79%) remained asymptomatic (mean follow-up 28 mo). Initial rest EF, ex EF or ΔEF did not distinguish the patients who developed symptoms from the other patients with -ΔEF. For all patients, LVEF decreased during serial studies both at rest (53 $\pm$ 8 to 50 $\pm$ 7%, $p < .001$) and during ex (46 $\pm$ 11 to 44 $\pm$ 10%, $p < .005$) but neither the initial ex EF or ΔEF were helpful in predicting these changes. In the 11 symptomatic patients, ex LVEF increased after operation (35 $\pm$ 10 preop, 54 $\pm$ 19% postop, $p < .001$); it failed to improve > 5% in only 3 patients with subnormal preop rest EF. Thus, 1) LVEF decreases during ex early in the course of AR, with 90% of asymptomatic patients with LVSD > 40 mm manifesting -ΔEF; 2) neither the initial ex EF nor ΔEF predicts which patients will develop symptoms over a 2 year period and does not predict deterioration in resting LVEF: and 3) depressed preop ex LVEF in symptomatic patients improves postop. These data suggest the ex LVEF has unproven prognostic value and should not yet be used alone to determine timing of operation in asymptomatic patients.

Publications: none

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 04032-01 CB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Radionuclide and Echo Study of Left Ventricular Function in Aortic Regurgitation.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

Robert O. Bonow	Senior Investigator,	CB, NHLBI
Douglas R. Rosing	Senior Investigator,	CB, NHLBI
Barry J. Maron	Senior Investigator,	CB, NHLBI
Stephen L. Bacharach	Physicist,	APS, NM, CC
Michael V Green	Chief,	APS, NM, CC
Stephen E Epstein	Chief,	CB, NHLBI

COOPERATING UNITS (if any)

Applied Physics Section, Nuclear Medicine Dept., CC, NIH

LAB/BRANCH
Cardiology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.5	.5	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Left ventricular sytolic function is an important determinant of long-term prognosis in patients with aortic regurgitation (AR). We studied 130 patients with AR to correlate assessment of LV systolic function by radionuclide angiography, (LV ejection fraction) and echocardiography (LV fractional shortening and systolic dimension). Radionuclide and echo assessment of LV systolic function correlated well at rest. In addition LV ejection fraction during exercise was also related to resting echo LV systolic dimension. During serial studies, echo LV systolic dimension was a stronger predictor of deterioration in resting LV function than was radionuclide ejection fraction. Thus, echo data provides important additional information to radionuclide assessment of LV function in the management of patients with AR.

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PHS-6040
(Rev. 2-81)

The left ventricular (LV) ejection fraction (EF) at rest and the echo LV systolic dimension (LVSD) at rest have important prognostic value in patients with aortic regurgitation (AR). Many patients with normal LVEF at rest develop abnormal LVEF with exercise (ex). To determine the relation between LVSD at rest and LVEF at rest and during ex, we studied 130 consecutive patients with AR (37 with and 93 without symptoms) with both echo and radionuclide gangiography. LV fractional shortening by echo correlated well with rest LVEF ($r = 0.75$). LVSD was strongly related to both rest and ex LVEF and the change (ΔEF) from rest to ex (* = $p < .01$):

LVSD (mm)	#	Rest EF	ExEF	ΔEF
< 40	28	56 ± 8% *	61 ± 11% *	5 ± 8 *
41 - 49	42	54 ± 8% *	50 ± 11% *	-4 ± 7 *
50 - 54	31	49 ± 8% *	41 ± 9% *	-8 ± 7 *
> 55	29	40 ± 10% *	32 ± 11% *	-8 ± 7 *

During serial studies performed in 70 asymptomatic patients (mean follow-up 28 mo), changes in both rest and ex LVEF with time were best predicted by the initial LVSD, with greatest changes occurring in patients with initial LVSD > 50 mm (52 ± 9 to 47 ± 18% for rest EF, $p < .005$, and 43 ± 8 to 40 ± 9% for ex EF, $p < .05$). These changes were not predicted by either the initial rest LVEF, ex LVEF, or ΔEF. Thus, 1) LVEF at rest, with ex, and ΔEF is significantly related to echo LVSD, and 2) LVSD is helpful in predicting deterioration of rest and ex LVEF. These data indicate that echo data provide important additional information to radionuclide addessment of LV function in the management of patients with AR.

Publications: none

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 04033-01 CB

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

The comparative cardiac effects of verapamil and nifedipine in the setting of congestive heart failure and ischemia in dogs.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Urquhart	Staff Fellow	CB, NHLBI
Other:	R.E. Patterson	Senior Investigator	CB, NHLBI
	S.E. Epstein	Chief, Cardiology Branch	CB, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Cardiology Branch

SECTION

Experimental Animal Physiology and Pharmacology Laboratory, Cardiology Branch

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.1	.1	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Verapamil and nifedipine are drugs which have the potential to depress myocardial function while also improving coronary artery blood flow and lowering blood pressure which decreases the work of the heart. We studied these two drugs in a model of congestive heart failure to see if either improved or worsened the function of the heart muscle and found that verapamil used in the usual clinical does caused deterioration in myocardial function while nifedipine improved function. These results suggest verapamil is more dangerous to use than nifedipine in the presence of congestive heart failure.

PHS-6040
(Rev. 2-81)

Project Description:

Since verapamil (V) and nifedipine (N) have potent vasodilator and negative inotropic effects. they could either improve or exacerbate congestive heart failure (CHF). Previous studies of normal or ischemic myocardium showed that V and N exert different myocardial effects relative to vasodilator potency. However, no studies exist relating to whether either (or both) of these drugs is dangerous to use in CHF. 14 studies were performed in 5 conscious dogs with previous total LAD occlusions. After dextran infusion to increase (↑) left atrial pressure (LAP), the CFX coronary artery was partially occluded and V or N administered. LV function was assessed by gated blood pool scintigraphy. Drug effects were compared at doses causing a decrease (↓) in mean arterial pressure (MAP) of 10%.

	LAP		HR		G-EF		I-EF	
	PO	Rx	PO	Rx	PO	Rx	PO	Rx
N 27 mm Hg		-18%	137	+ 35%	.29	+ 21%	.18	+ 22%
		p <.02		p = .001		p =.01		p < .001
V 26 mm Hg		+61%	148	- 4%	.35	- 6%	.21	- 6%

HR = heart rate, EF = ejection fraction, G = global, I = ischemic region, PO = postocclusion. With larger drug doses (20 ↓ in MAP), N led to a further ↓ in LAP, however, the ↑ in LAP caused by lower doses of V was reversed so that LAP returned to control values (26 mm Hg). Thus, in the presence of LV failure an ischemia, cardiac function improves with N (as manifest by ↑ LVEF and ↓ LAP), but deteriorates with V (as evident by ↑ LAP), although at higher doses the afterload reducing effect of V overcomes the myocardial depressant effect. These results suggest V is more dangerous to use than N in the presence of LV failure and ischemia.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 04034-01 CB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Manganese-52m: A new myocardial perfusion imaging agent for positron emission tomography that does not require an on-site cylotron.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Urquhart	Staff Fellow	CB, NHLBI
Other:	R.E. Patterson	Senior Investigator	CB, NHLBI
	R. Aamodt	Chief, Whole Body Count Section	DNM:CB, NHLBI
	E. Speir	Chemist	CB, NHLBI
	R. Keller,	Senior Investigator,	DNM:CB, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Department of Nuclear Medicine Clinical Center

SECTION
Cardiology Branch

INSTITUTE AND LOCATION
Experimental Animal Physiology and Pharmacology Laboratory, Cardiology Branch

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
NHLBI, NIH, Bethesda	Maryland 20205	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Positron emission tomography (PET) has tremendous potential as a method of imaging blood flow to the heart in three dimensions. Its use has been limited by the need for an on-site cylotron. We tested the physiological characteristics of manganese 52m which is available from a generator that can be shipped conveniently. We found it was as good an indicator of myocardial blood flow as the standard tracers used, thallium 201 and rubidium 82. Therefore, manganese 52m will be a useful radionuclide for use in myocardial scanning with the PET scanner.

PHS-6040
(Rev. 2-81)

Project Description:

The great potential of myocardial perfusion imaging (MPI) by positron emission tomography (PET) has been limited by need for an on-site cyclotron to produce N-13 or by need for a special high speed PET system to image the ultra-short t 1/2 of Rb-82 (75 sec). Our purpose was to test the physiological characteristics of Mn as an indicator of myocardial blood flow (MBF) and the speed of its clearance from blood to tissue by comparing Mn to other diffusible indicators. Mn-52m is available from a generator (Fe-52) which can be shipped conveniently (t 1/2 = 8 hr) and Mn-52m has a t 1/2 (20 min) which permits repear imaging on the same day. We compared Mn with Rb and Tl-201 in 5 dogs with acute coronary occlusion receiving isoproterenol to produce a wide range of MBF (by Ce-141 microsphere) from 0.01 to 4.0 ml/min/g. Dogs were sacrified 4 min after simultaneous injection of all 4 indicators into the left atrium, reference arterial blood and myocardial samples were counted. The correlations in myocardal samples from each dog for Mn activity vs. MF were as high (r = .85 mean, and .69 - .99 range) as between Tl (r = .90, .80 - .98) or Rb (r = .89, .77 - .99) vs MBF. All diffusible indicators had similar positive intercept values when MBF = 0. In another dog the clearance form blood to tissue of each indicator was similarly rapid (t 1/2 = 25 -40 sec). We conclude that Mn-52m appears to be a promising radionuclide for MPI because of its availability from a generator, convenient t 1/2, and rapid clearance from blood to tissue. Most important, Mn deposits in tissue as a linear function of blood flow as well as Tl-201 and Rb.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 04035-01 CB

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Patterns of inheritance in Hypertrophic Cardiomyopathy: An assessment by M-mode and 2-D Echocardiography

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Barry J. Maron	Senior Investigator	CB, NHLBI
Other:	P.R. Nichols	Guest Worker	CB, NHLBI
	L.W. Pickle	Senior Staff Fellow	NCI
	J.J. Mulvihill	Senior Investigator	NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Cardiology Branch

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.99	.88	.77

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Hypertrophic Cardiomyopathy may be genetically trasmitted or "Sporadic (or non-genetic). This implies that the phenotypic expression of the disease may have multiple etiologies.

157

PHS-6040
(Rev. 2-81)

Project Description:

Hypertrophic cardiomyopathy (HCM) shows obvious genetic transmission in some families. However, the frequency with which HCM is familial, the precise mode of inheritance, and whether it has multiple etiologies are largely unresolved issues. To determine more definitively patterns of inheritance of HCM, 298 patients from 70 families were studied by M-mode and 2-D echo. Only 22% of first degree relatives were affected, of which one-fifth were considered "subclinical". Inspection of pedigrees showed the disease was familial with autosomal dominant pattern in 39 (56%) but sporadic in 31 (44%). Probands commonly showed symptoms (81%), subaortic obstruction (53%), diffuse and marked LV hypertrophy (51%); in contrast, affected relatives were asymptomatic (72%), without obstruction (94%) and had less diffuse LV hypertrophy (60%; $p < 0.001$). Patients with the familial or sporadic forms did not differ in phenotypic expression of HCM. Hence, in HCM: (1) gentic transmission is common, usually with an autosomal dominant pattern; (2) sporadic occurrence is present in a substantial proportion of families; (3) a variety of phenotypic expressions occur which may have genetic as well as nongentic etiologies; (4) pattern of inheritance demonstrated in a given family.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 04036-01 CB

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Relation of ECG Abnormalities and Patterns of Left Ventricular Hypertrophy identified by 2-D Echocardiography in patients with Hypertrophic Cardiomyopathy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Barry J. Maron	Senior Investigator	CB, NHLBI
Other:	J.K. Wolfson	Guest Worker	CB, NHLBI
	E. Ciro	Guest Worker	CB, NHLBI
	P. Spirito	Guest Worker	CB, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Cardiology Branch

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.11	.1	.01

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In patients with hypertrophic cardiomyopathy ECG abnormalities and morphologic abnormalities may both show great variability. However, the ECG pattern of left ventricular hypertrophy is usually an indicator of diffuse and marked increase in mass.

PHS-6040
(Rev. 2-81)

Project Description:

Scalar ECGs show a wide spectrum of alterations in patients with hypertrophic cardiomyopathy (HCM), although the significance of these changes is often unclear. Two-dimensional echocardiography (2-D echo) has alos demonstrated a variety of patterns of left ventricular hypertrophy (LVH) in HCM. To determine whether distribution of LVH on 2-D echo influences ECG patterns. LVH was assessed by 2-D echo in 153 patients with HCM and compared to the ECG. Most common EC alterations were ST-T changes (61%), LVH (47%), Q waves (25%). LVH on ECT was significantly more common in patients with the most extensive distribution of LVH involving substantial portions of both ventricular septum an LV free wall (51/69, 74%), than in patients with more limited distribution with HCM and normal ECG (13/23) had mild localized LVH. Q waves were more common in patients without hypertrophy of the basal ventricular septum (15/27, 56%) than in patients with basal septal hypertrophy (23/126, 18%; $p < 0.001$). Hence, in HCM: (1) LVH pattern on ECG, although present in only about one-half of patients is relatively sensitive (i.e. 74%) marker for diffuse, extensive LVH; (2) Q waves connot be explained by septal hypertrophy alone; (3) a noraml ECG is most commonly a manifestation of mild localized LVH.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 04037-01 CB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Heterogenous Expression of the Morphologic Marker in Genetically Transmitted Hypertrophic Cardiomyopathy: A Two-Deminsional Echocardiographic Study.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Barry J. Maron	Senior Investigator	CB, NHLBI
Other:	E. Ciro	Guest Worker	CB, NHLBI
	P F. Nichols	Guest Worker	CB, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Cardiology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.1	.1	.01

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

When determined by two-Dimensional echocardiography the morphologic expression of hypertrophic cardiomyopathy is variable, even in closely related patients.

PHS-6040
(Rev. 2-81)

Project Description:

Hypertrophic cardiomyopathy (HCM) is often genetically transmitted as an autosomal dominant trait and shows a variety of patterns of left ventricular hypertrophy (LVH) that may be identified by two-dimensional echo (2 DE). To determine whether distribution of LVH is morphologically similar in affected first degree relatives, 40 index cases with HCM and 66 affected relatves, were studied by 2 DE. Presence or absence of hypertrophy was assessed in 10 segments of LV wall by analyzing real-time echo in 3 cross-sectional planes. Distribution of LVH was compared in all possible combinations of pairs of relatives and then the data was described in the following way: (1) indentical (Hypertrophy involving the same segments of LV in each relative; (2) similar (hypertrophy involving > 9 of the same segments); (3) dissimilar (involving < 8 of the same segments). Distribution of LVH among 105 familial relations was identical in 8 (7%), similar in 24 (23%), but dissimilar in 73 (70%); $p < 0.001$). Morphologic dissimilarities among relatives were not due to differences in symptomatic state or magnitude of subaortic gradient. Hence, in the genetically transmitted form of HCm, 2-D echo demonstrates that morphologic expression of the disease in closely related patients is variable and usually markedly dissimilar.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 04038-01 CB

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Disease Progression in Hypertrophic Cardiomyopathy due to Persistent Changes in the Magnitude of LV Outflow Obstruction.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Barry J. Maron	Senior Investigator	CB, NHLBI
Other:	E. Ciro	Guest Worker	CB, NHLBI
	S.E. Epstein	Head, Cardiology Branch	CB, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Cardiology Branch

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.11	.1	.1

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Patients with hypertrophic cardiomyopathy may show marked changes in outflow gradient with time. Such increases or decreases in gradient are usually associated with clinical deterioration.

PHS-6040
(Rev. 2-81)

Project Description:

Dynamic subaortic obstruction is a prominent feature of many patients with hypertrophic cardiomyopathy (HCM). Although spontaneous regression or appearance of LV outflow obstruction has been known to occur, the significance of this hemodynamic alteration has not been established. Hence, the clinical course of 23 nonoperated patients with HCM who showed substantial spontaneous reduction or increase in magnitude of subaortic obstruction were analyzed. Variations in hemodynamic state were shown by serial catheterizations in 21 patients and by echo in 2 patients. Period of time elapsed between the 2 evaulations ranged 1 - 21 years. Reduction or loss of outflow obstruction was noted in 8 patients; increase in obstruction occurred in 15 patients. Of the 8 patients with reduction or loss of obstruction, 6 (75%) deteriorated clinically and 2 (25%) were stable. Of the 15 patients with increased gradient, 10 (66%) showed clinical deterioration and 5 (34%) were unchanged. Atrial fibrillation appeared responsible for increased symptoms in 3 patients with loss of obstruction and 1 patient with increased gradient. We conclude : (1) substantial changes in magnitude of subaortic obstruction occur in some patients with HCM as part of the natural history of the disease; (2) such hemodynamic alterations are usually associated with progression of the disease and clinical deterioration.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 04039-01 CB

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Two-Dimensional echo assessment of LV outflow tract cross-sectional area in hypertrophic cardiomyopathy: Diagnostic utility and role in producing subaortic obstruction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Barry J. Maron	Senior Investigator	CB, NHLBI
Other:	P. Spirito	Guest Worker	CB, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Cardiology Branch

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.45	.4	.05

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The size of the left ventricular outflow tract is an important determinant of whether subaortic obstruction is present in patients with hypertrophic cardiomyopathy. Also, the size of the outflow tract may permit us to distinguish patients with hypertrophic cardiomyopathy from normal.

PHS-6040
(Rev. 2-81)

Project Description:

Hypertrophic cardiomyopathy (HCM) may or may not be associated with subarotic obstruction. Obstruction results from systolic anterior motion of the mitral valve (SAM) into the LV outflow tract; however, the determinants of SAM are not completely understood. To define the role of LV outflow tract orifice size in determining subaortic obstruction, 66 patients with HCM and 16 normal controls were studied by 2-D echo. LV outflow tract area (LVOA) was measured in short-axis view in stop-frame mode. Patients with HCM were classified into 3 groups based on catheterization gradient: Group I obstruction (21 patients), Group II provacable (14 patients), Group III nonobstructive (31 patients). LVOA was abnormally small in patients with HCM (4.6 ± 2 cm^2) compared to normals (10 ± 1 cm^2, $p < 0.001$). LVOA was smallest in Group I (2.6 ± 0.7 cm^2), and largest in Group III (6 ± 1 cm^2, $p < 0.001$) 20/21 Group I patients showed LVOA < 4 cm^2, while 29/31 Group III patients had LVOA > 4 cm^2. Group II patients were intermediate with an LVOA of 4.6 ± 1 cm^2. Hence, the size of the LV outflow tract appears to be of major pathophysiologic significance in producing subaortic obstruction in HCM. Its measurement also may be of diagnostic utility in distinguishing patients wtih HCM from those patients without heart disease in whom echocardiographic assessment of LV hypertrophy is equivocal.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 04040-01 CB

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Prevalence and Significance of ST Segment response to exercise in Athletes.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Barry J. Maron	Senior Investigator	CB, NHLBI
Other:	P. Spirito	Guest Worker	CB, NHLBI
	R.O. Bonow	Senior Investigator	CB, NHLBI
	S.E. Epstein	Head, Cardiology Branch	CB, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Cardiology Branch

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.11	.1	.01

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

About 10% of competitive athletes may have an abnormal ECG stress test which is not indicative of underlying heart disease. This is an important point in screening athletes for heart disease.

PHS-6040
(Rev. 2-81)

Project Description:

ECG exercise stress testing is employed widely to detect underlying cardiovascular disease in asymptomatic individuals. However, the frequency and significance of an abnormal ST segment response to exercise in athletes with increased LV mass induced by physical conditioning are unknown. To determine efficacy of treadmill ECG testing in the cardiovascular evaluation of athletes, 75 male isometrically trained athletes without evidence of heart disease (mean age 21 years) and 48 nonathletic normals were studied by M-mode echo and exersice ECG. Ventricular septal and posterior free wall thicknesses and calculated LV mass were significantly greater in athletes than in controls. An abnormal ST segment response to exercise was present in 7 (9%) of the 75 athletes, five of whom had "physiologic" LVH with mass greater than the 95th percentile of controls; 5/7 had rest-exercise radionuclide studies, each of which was normal. An abnormal treadmill exercise test was also present in (6%) of 48 controls. Hence, abnormal exercise tests occur commonly in athletes as well as nonathletes. The fact that almost 10% of isometrically trained athletes exhibit "false positive" exercise ECGs suggests that the ECG stress test has major limitations as a screening test for underlying cardiovascular disease in athletes.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 04041-01 CB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Prospective Evaluation of the Cardiotoxicity of Anticancer agents.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Sebastian T. Palmeri	Expert Consultant	CB, NHLBI
	Robert O. Bonow	Senior Investigator	CB, NHLBI
Other:	Charles E. Myers	Chief, Clinical Pharmacology Br.	CP, NCI
	Steven A. Rosenberg	Chief, Surgical Branch	Surg, NCI
	Teresa D'Angelo	Nurse	Surg, NCI
	Steven L. Bacharach	Physicist, APS	NM, CC
	Michael V. Green	Chief, APS,	NM, CC
	Jean Jenkins	Nurse	CP, NCI

COOPERATING UNITS (if any)
Surgical Branch, NCI
Clinical Pharmacology Branch, NCI
Department of Nuclear Medicine, Applied Physics Section Clinical Center

LAB/BRANCH
Cardiology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.1	.1	.01

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Adriamycin and cytoxan are two chemotherepeutic agents widely used in the treatment of malignant tumors. We prospectively followed 25 patients by serial radionuclide angiography to determine the incidence and severity of the cardiac damage caused by these agents. Only patients free of preexisting heart disease with normal resting LV ejection fractions were studied. After treatment with adriamycin and cytoxan only 4 patients developed clinical evidence for heart failure. By radionuclide testing, however, 24 patients showed some evidence for cardiac damage. Twelve patients developed subnormal resting LV ejection fractions. These data appear to indicate that in the absence of underlying heart disease it is uncommon for adriamycin or cytoxan to cause clinically significant LV dysfunction. Furthermore, serial radionuclide angiography appears to be a sensitve method for following patients receiving these agents.

Project Description:

Clincial left ventricular (LV) dysfunction develops in many patients who receive adriamycin, but the frequency with which adriamycin produces depression of LV function is unknown. We studied 25 consecutive patients (mean age 43, range 15 - 62) by radionuclide angiography before and after receiving adriamycin (mean total dose 521 mg/m^2, range 480 - 55 mg/m^2) and cytoxan (range 4800 - 5500 mg/m^2). All patients had normal resting LV ejection fractions before their chemotherapy (56 $\pm$ 9%). After chemotherapy the LV ejection fraction was significantly reduced (45 $\pm$ 9%; $p < .0005$), and 12 patients (48%) developed subnormal resting LV ejection fractions. The LV ejection fraction was reduced > 5% compared to the pre-treatment level in 19 patients. Moreover, in the 6 patients in whom the LV ejection fraction at rest was not reduced, it was reduced during exercise > 9% in 5 patients. Although combined adriamycin and cytoxan produced clinical LV dysfunction in only 4 patients (17%), by prospective analysis these agents depressed LV function at rest in 76% and impaired LV function during exercise in another 20%. Hence, 96% of patients manifested negative effects on LV function. Nonetheless, these data indicate that in the absence of preceeding heart disease it is uncommon for clinically significant LV dysfunction to develop after combined adriamycin and cytoxan chemotherapy. Furthermore, serial radionuclide angiography appears to be a sensitive method for following patients, and may permit selected patients to safely receive a higher total dose of these anticancer agents.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 04042-01 CB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Effect of Verapamil in preventing coronary artery restenosis after successful percutaneous transluminal coronary angioplasty.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. R. Rosing	Head, Cardiovascular Diagnosis	CB, NHLBI
Other:	K. M. Kent	Clinical Associate Prof of Med Georgetown University	
	Carolyn Ewels	Biologist	CB, NHLBI
	S. E. Epstein	Chief, Cardiology Branch	CB, NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.3	.2	.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Coronary artery restenosis after precutaneous transluminal coronary angioplasty (PTCA) was a common finding after a successful precedure. The calcium channel blodking drug verapamil was randomly administered pool successful PTCA to see if the restenosis could be reduced. The administration of verapamil has, so far, not reduced the occurrence of restenosis.

PHS-6040
(Rev. 2-81)

Project Description:

During our initial experience with percutaneous transluminal coronary angioplasty, a relatively high restenosis rate of 35% occurred in the first 17 successful procedures. After this experience, we routinely started to administer verapamil for two to six months of follow-up to patients who had successful results. Doses used were usually 360 - 480 mg/day. After the next 32 successful dilatations, the restenosis rate was only 22%. Thus, we began a randomized, but non-blinded study administering either verapamil or no medication to patients for the first two months after a successful PTCA. Thus far, 15 patients have received verapamil and nine no medication. Four of the 15 (27%) on verapamil have had the occurrence of restenosis, while four of the nine (44%) on no medication have restenosis. This difference is not statistically significant at the present time.

Publication: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 04043-01 CB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Randomized Trial of Percutaneous Transluminal Coronary Angioplasty versus Coronary Artery Bypass Surgery in Patients with Multiple Vessel Disease.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. R. Rosing	Head, Cardiovascular Diagnosis	CB, NHLBI
Other:	K. M. Kent	Clinical Associate Prof of Med Georgetown University	
	Carolyn Ewels	Biologist	CB, NHLBI
	Richard O. Cannon	Clinical Associate	CB, NHLBI
	Rita M. Watson	Clinical Associate	CB, NHLBI
	Charles L. McIntosh	Senior Surgeon	CB, NHLBI
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	S. E. Epstein	Chief, Cardiology Branch	CB, NHLBI

COOPERATING UNITS (if any)

Surgery Branch

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.3	.2	.1

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
In order to determine if percutaneous transluminal coronary angioplasty can provide as much symptomatic relief as coronary artery bypass surgery in patients with coronary artery disease. A randomized trial was undertaken examing the relative success of the two interventions. So far the number of patients accessed into the study is too small to draw any conclusions.

PHS-6040
(Rev. 2-81)

Project Description:

A number of patients undergo coronary artery bypass surgery and have one or more occluded grafts, yet have a good symptomatic response and thus a good clinical result. As a result, it was postulated that patients who have multiple vessel disease in whom one or more vessels may have lesions which are suitable for a percutaneous transluminal coronary angioplasty (PTCA) may also receive a good clinical result from the procedure. We thus began a randomized study of patients who had multiple vessel disease and who had at least one vessel with a lesion that could be approached with a PTCA procedure. Patients fulfilling this definition who were felt to be candidates for bypass surgery either because of their anatomy or due to symptoms refuactory???? to medical treatment were randomized into either the surgical or PTCA groups. Thus far, four patients have been designated for surgery and two for PTCA treatment. All patients are alive, but the two who initially ahd PTCA have required subsequent surgery because of restenosis or persistent symptoms. It has been very difficult to access patients into this study for several reasons including an unwillingness on the part of a patient to take the chance of being randomized into the surgical group. As a result the approach to answering the posed question will probably have to be changed.

Publication: None

Annual Report of the
Laboratory of Cell Biology
National Heart, Lung, and Blood Institute
October 1, 1981 to September 30, 1982

In this year's summary of the activities of the Laboratory of Cell Biology we will discuss accomplishments in the following areas: (1) the biochemistry of muscle contraction; (2) the polymerization of actin; (3) the characterization of myosins from nonmuscle cells; (4) the biochemistry of microtubules; (5) energy transduction in biological membranes; and (6) membrane movements in endocytosis. There has also been an important personnel change in that Dr. Lois Greene has been converted to permanent status.

(1) Biochemistry of Muscle Contraction: Dr. Evan Eisenberg and his colleagues have made progress in four major areas of research during the past year. First, they have obtained further evidence for their kinetic model of the actomyosin ATPase activity. The major area of disagreement with other groups remains the nature of the rate-limiting step in the actomyosin ATPase cycle. Dr. Eisenberg's group had already shown that the rate-limiting step cannot be P_i release. Therefore, the remaining possibilities are the ATP hydrolysis step, itself, or a step which follows the ATP hydrolysis step. Using a preparation of myosin subfragment-one covalently cross-linked to F-actin, they have now shown that the ATP hydrolysis step occurs too fast to be rate-limiting. Therefore, the rate-limiting step must follow ATP hydrolysis but occur before P_i release. This rate-limiting step is important because it may well control the velocity of muscle contraction.

They have also tested whether their actomyosin kinetic model fits the data obtained with smooth muscle S-1. Their results show that their kinetic model does fit the data but there is one major quantitative difference between skeletal and smooth muscle S-1. The binding of smooth muscle S-1 to actin shows much less salt dependence than the binding of skeletal muscle S-1. This means that under physiological conditions smooth muscle myosin binds almost 10 times more strongly to actin than does skeletal muscle myosin, and this may account for the greater force production of smooth muscle compared to skeletal muscle.

A second major area of research has been the mechanism of muscle relaxation. Previously, Dr. Eisenberg showed that, in skeletal muscle, troponin-tropomyosin does not cause relaxation by blocking the binding of the cross-bridge to actin as was suggested by the popular steric blocking model. Rather, troponin-tropomyosin apparently acts by blocking a step which occurs after the binding of the cross-bridge perhaps a step involving rotation of the cross-bridge on actin, a step which is thought to be associated with P_i release and to occur after the initial attachment step. Their initial data on this point had been obtained using single-headed myosin subfragment-one. Other workers had suggested that perhaps the two-headed fragment of myosin, heavy meromyosin would act differently but Dr. Eisenberg's group has now confirmed that the same behavior occurs with heavy meromyosin as with subfragment-one.

Dr. Eisenberg and his associates have also extended their investigation of muscle regulation to smooth muscle. Here, regulation occurs in a very different manner than with skeletal muscle. Rather than Ca^{2+} binding to troponin-tropomyosin causing relaxation, relaxation in smooth muscle is caused by dephosphorylation of myosin. Nevertheless, they have shown that here too, regulation does not work by controlling the binding of myosin to actin but rather by blocking a step which occurs after the myosin binds to actin, perhaps as in skeletal muscle, the rotation of the cross-bridge. Thus, with two quite different mechanisms of muscle relaxation, it is not the binding of myosin to actin but a subsequent step which is blocked to prevent the cross-bridge cycle from occurring.

Since Dr. Eisenberg's mechanism of skeletal muscle relaxation predicts that cross-bridges will not be blocked from binding to actin in relaxed muscle, they undertook a collaborative study to determine whether evidence of attached cross-bridges could be found in relaxed skinned rabbit muscle fibers. Their results showed that the cross-bridges are indeed attached at very low ionic strength, as shown by the presence of a transient stiffness when the muscles are pulled very rapidly in the msec time range. With slower stretches, stiffness was not detected, suggesting that the cross-bridges very rapidly attach to and detach from actin in relaxed muscle at least at very low ionic strength. Whether cross-bridges are also attached under physiological conditions is presently being investigated.

A third major area of research has been a continuing investigation into the mechanism of action of troponin-tropomyosin in blocking the binding of S-1 to actin in the absence of ATP. Their major effort over the past year has been to investigate the role of troponin in this effect by comparing tropomyosin alone with troponin-tropomyosin. The results show that, as far as the binding of S-1 is concerned, in the presence of Ca^{2+}, troponin-tropomyosin and tropomyosin alone have almost the same effect. Both cause very slight cooperative binding of the S-1 to actin. On the other hand, in the absence of Ca^{2+}, troponin-tropomyosin causes a much greater response than tropomyosin. This suggests that the major effect of troponin occurs in the absence of Ca^{2+}, and in the presence of Ca^{2+} it has little further effect on the binding of S-1 to actin. Experiments are now underway to determine whether troponin plays a similar role in the acto·S-1 ATPase activity, affecting this activity only in the absence of Ca^{2+} and not in the presence of Ca^{2+}.

Finally, studies on the effect of troponin-tropomyosin on the interaction of S-1 and actin have provided a new tool to determine whether different S-1 states bind to actin with different orientations. Most models of muscle contraction suggest that a conformational change takes place in the acto·S-1 complex when the products of ATP hydrolysis are released. More specifically, it has been proposed that the actin-bound cross-bridge rotates from a 90° attachment angle to a 45° attachment angle when P_i is released. Previously, Dr. Eisenberg found that the binding of S-1·ATP and S-1 to actin are affected by troponin-tropomyosin in completely different ways -- the binding of S-1 is blocked in a cooperative manner while the binding of S-1·ATP is unaffected. They now find that this is not simply because the binding of S-1·ATP is weaker than the binding of S-1. By raising the salt, S-1·ADP or S-1·AMP-PNP can be made to bind just as weakly as S-1·ATP yet troponin-tropomyosin still blocks

their binding to actin in a cooperative manner. This suggests that S-1·ATP binds to actin with a different orientation than S-1 and explains why tropomyosin cannot affect its binding. They have now shown that S-1 can be modified with a reagent which cross-links two SH groups internally and when this is done the modified S-1 binds very weakly to actin just like S-1·ATP. Furthermore, the binding of this modified S-1 is essentially unaffected by troponin-tropomyosin which suggests that it binds to actin with the same orientation as S-1·ATP. Thus, it may be an analogue of the "90°" attached cross-bridge and since it binds in the absence of ATP, its structure may be able to be determined by electron microscopy.

(2) The Polymerization of Actin: According to accepted theory, actin polymerizes in several stages: an initial slow phase in which several actin monomers form a small nucleus, a rapid phase in which the nucleus rapidly elongates at both ends to form long filaments, and a final phase in which the filament lengths redistribute to their final equilibrium number/length distribution. In research conducted by Dr. Korn and his colleagues, polymerization has been monitored by following the increase in fluorescence of a pyrenyl probe attached to cysteine 374 which allows the entire process to be followed without perturbing the system. Computer fitting of the rates of polymerization as a function of actin concentrations led to the unambiguous conclusion that, under all conditions of polymerization examined, the size of the nucleus is four (within the limits of the mathematical model used). There is no necessity to introduce a term to account for the possibility of fragmentation of filaments. Polymerization of actin with a bound Ca^{2+} is slower than when the actin has a bound Mg^{2+}. Kinetic analysis has shown that this difference is due almost entirely to differences in the rates of nucleation and that Ca-actin and Mg-actin have very similar rates of elongation. By changing ionic conditions, such as the concentrations of KCl, $MgCl_2$ and $CaCl_2$, the time to reach complete polymerization can be varied over a wide range. It has been shown that most of this difference is due to changes in the rates of nucleation although there are significant, albeit much smaller, changes in the rates of elongation and dissociation.

About one molecule of ATP is hydrolyzed for every molecule of actin that polymerizes. At steady state, when polymerization is complete, the hydrolysis of ATP continues. The mechanism of the steady-state hydrolysis of ATP is of interest. Dr. Brenner and Dr. Korn have shown that, in 0.5 mM $MgCl_2$, four molecules of ATP are hydrolyzed for each actin monomer that is converted to actin polymer. The equilibration of actin monomer and actin polymer is followed by adding tracer quantities of pyrenyl-actin monomer at steady-state and monitoring the increase in fluorescence. The kinetics of the complete equilibration of monomer and polymer should provide insight into the mechanism of their interconversion. Analysis of the data is complicated by the fact that the actin filaments are of different lengths and a clear distinction has not yet been possible between the two most likely mechanisms: treadmilling (in which there is net addition of actin monomers at one end of the filaments and equal net loss of actin monomers from the other end) and exchange-diffusion simultaneously at both ends of the filaments. The fact that the rate of equilibration is greatly inhibited by cytochalasins B and D (which bind specifically to one end of the filaments) and when ADP is substituted for ATP (treadmilling should and exchange-diffusion should not require ATP) suggest that the mechanism is treadmilling.

When steady-state equilibration of monomeric and polymeric actin is followed in the presence of $CaCl_2$ (rather than $MgCl_2$) much less than complete equilibration is obtained and the process is much slower and the kinetics may suggest, as anticipated for these conditions, that exchange-diffusion is the mechanism. Similarly, exchange-diffusion probably accounts for the interconversion of monomer and polymer in KCl solutions.

The polymerization of actin dimers covalently cross-linked by phenylenebismaleimide has been studied further this year by Dr. Korn's group. In this case, there seems to be no requirement for a nucleation step but Mg-actin dimer does polymerize more rapidly than Ca-actin dimer, as was observed for the native monomer. When covalently cross-linked actin dimers polymerize, the hydrolysis of ATP is very much slower than the polymerization process. As a result, only about 15% of the ATP is hydrolyzed when polymerization is complete. Hydrolysis of the ATP bound to one of the two ATP sites on the dimer proceeds fairly rapidly but the hydrolysis of the ATP bound to the second site requires many hours even though polymerization is complete in minutes. These data indicate that, for cross-linked actin dimer (and inferentially for native monomer as well), polymerization and ATP hydrolysis are separate events with F-actin-ATP being formed as a result of polymerization and hydrolysis of ATP occurring at the polymeric stage. These data are in agreement with previous hypotheses of Dr. Korn's group that both monomeric and polymeric actin alone can catalyze the hydrolysis of ATP and lends some support to the postulations that the role of ATP hydrolysis is either to provide a means for regulating the polymerization process or possibly to influence the properties of the F-actin.

(3) The Characterization of Nonmuscle Myosins: Dr. Korn's group has continued their studies of the three myosin isoenzymes from Acanthamoeba castellanii which, as a result of the work of this group and of Dr. Tom Pollard at The Johns Hopkins University, are probably the best characterized nonmuscle myosins. The kinase that phosphorylates the heavy chain of myosin IA and IB has been purified to near homogeneity and shown to catalyze the addition of one phosphate per heavy chain. This phosphorylation is required for expression of actin-activated ATPase activity. The $K_{binding}$ and K_{ATPase} for F-actin have been shown to be about 1 μM for phosphorylated myosin IA and IB and the $K_{binding}$ appears also to be 1 μM for the dephosphorylated forms of the enzymes. It seems likely, therefore, that the differences in enzymatic activity between the phosphorylated and unphosphorylated forms of the enzymes are differences in V_{max} and are not due to differences in the abilities of the enzymes to bind to F-actin. The catalytic activity of the dephosphorylated term is too low to measure. The $K_{binding}$ is much tighter than the binding of F-actin to muscle subfragment-one. Sedimentation-equilibrium analysis confirms that both myosin IA and IB are monomers of molecular weights about 180,000 and circular dichroism suggests that neither has more than about 5% α-helix confirming the previous evidence that they are globular proteins.

With myosin II, it is the dephosphorylated form of the enzyme that has actin-activated ATPase activity and the phosphorylated form that is enzymatically much less active. Dr. Korn and his associates have found that the difference in enzymatic activity is almost entirely a consequence of V_{max} since the two forms of the enzyme have very similar values of K_{ATPase} and $K_{binding}$ for F-actin ($K_{binding}$ is, however, significantly lower for the dephosphorylated

form) and differ 15-fold in V_{max}. Dephosphorylated myosin II forms filaments that are more stable to the dissociating effects of KCl and MgATP than are the filaments formed by phosphorylated myosin II. Thus, it now appears that the dephosphorylated form of myosin II shows enhanced self-association, enhanced association with F-actin and a higher V_{max} for the actin-activated ATPase activity than the phosphorylated myosin II.

Two other studies worth mentioning are the partial purification of a phosphatase that removes all six phosphates from phosphorylated myosin II and the purification to homogeneity and partial characterization of Acanthamoeba calmodulin in collaborative studies with Dr. Claude Klee, NCI.

(4) Biochemistry of Microtubules: Dr. Martin Flavin has initiated studies on the phosphorylation of MAP_2. MAP_2 is the predominant protein (mol. wt. 300,000 by SDS-PAGE) in vertebrate brain that coassembles in vitro in constant proportion to tubulin to form microtubules and promotes in vitro assembly of microtubules of pure tubulin. Current evidence suggests MAP_2 may be abundant only in dendritic processes of non-primate vertebrate brain cells. This very restricted distribution suggests a specific role in nerve function, to which there is so far no clue. However, MAP_2 accounts for such a large fraction of total protein phosphorylation in brain that investigation of it from this standpoint can be justified in its own right. Others have shown that at least 1/3 of all the cAMP-dependent protein kinase in brain is firmly bound to MAP_2; in fact, so far only exposure to cAMP has allowed dissociation of native kinase. MAP_2 could function to locally sequester the kinase, which when released would phosphorylate something else. But the obvious starting point in this new project is the well-known phosphorylation of MAP_2 itself.

Dr. Flavin's initial projects have been: (1) to purify a protein phosphatase active toward MAP_2; (2) to prepare maximally phosphorylated and dephosphorylated MAP_2 and compare their assembly-promoting properties; (3) to characterize the MAP_2 sites accessible to different kinases and phosphatases.

As phosphatase substrates, they prepared ^{32}P-labeled histone and ^{32}P-labeled MAP_2. The latter was prepared by autophosphorylation of tubulin-associated MAP_2. The product contained 10 to 12 moles phosphate per 300,000 g protein (the highest value reported before now had been 6. Various commercially available alkaline and acid phosphatases were found to rapidly proteolyze MAP_2 even in the presence of all available protease inhibitors.

In crude brain supernatant, fluphenazine (an inhibitor of calmodulin-dependent proteins) partially inhibited dephosphorylation of MAP_2. Calcineurin has recently been identified as a calmodulin-dependent protein phosphatase. Since calcineurin and MAP_2 are both at least 20 times more abundant in brain than in other tissues, Dr. Flavin's group is currently investigating whether this phosphatase acts on MAP_2 with some degree of specificity.

Meantime, they have purified the non-specific, alcohol-stable protein phosphatase catalytic subunit from brain. By modification of published procedures, it has been purified 600-fold in 22% yield. The following species of MAP_2 were then prepared and purified: (1) unmodified; (2) maximally autophosphorylated

which contained 10 newly introduced phosphate residues; (3) phosphorylated MAP_2 treated with purified phosphatase which removed 85% of the ^{32}P. Aliquots of each of these MAP_2 preparations were then maximally phosphorylated with added purified cAMP kinase catalytic subunit (the enzyme from beef heart muscle, which has been shown to be identical with that complexed with brain MAP_2). Both samples (1) and (3) could accept 10 moles of P/mole, and PAGE analysis showed all the ^{32}P to be in MAP_2. This suggested that MAP_2 as isolated from tubulin was completely dephosphorylated, and that no further sites had been exposed by phosphatase treatment. But sample (2) could also accept eight more phosphates for a total of 18. At this point, it appears that a different set of sites may be exposed in MAP_2 that has been separated from tubulin and/or heated to 100°C.

Tubulin assembly was promoted by sample (2) to a normal extent but at a much reduced rate. However, sample (3) was incompetent, indicating that it was not just reconstituted sample (1), but had been altered in another unidentified way.

(5) Energy Transduction in Biological Membranes: Energy liberated by respiration is transduced into an electochemical potential for protons ($\Delta\bar{\mu}H^+$). This energy form has two components, a membrane potential ($\Delta\Psi$) and a proton gradient (ΔpH); both of which are difficult to measure. Dr. Hendler has developed a system which measures both components easily and instantly. The system uses specific electrodes to assay a probe for membrane potential (TPP^+) and a probe for ΔpH (salicylic acid). They have designed and built these electrodes. The raw electrode signals must go through a series of complex computations before $\Delta\Psi$ and ΔpH are produced. These include digital filtering, corrections for electrode response times, and the solution of logarithmic expressions which produce the desired quantities. In addition to computing $\Delta\Psi$, ΔpH and their sum which is $\Delta\bar{\mu}H^+$, the same system follows, computes and reports, the rate of oxygen consumption, the rate of proton extrusion and the ratio of these which is the real time H^+/O ratio. In building this system custom electronic circuits, mathematical processing techniques and a large variety of computer software were created. The system is essentially completed and additional refinements will be added as needed. In the early applications of the system, they find that maximal $\Delta\bar{\mu}H^+$ can be formed virtually instantly when E. coli cells are exposed to minute quantities of oxygen (i.e. >99% anaerobic). Once formed, the energy lasts long after the last trace of oxygen has disappeared. H^+/O ratios measured by Dr. Hendler's technique show that much higher values are attained then have been reported in the literature. These high values are transient but nonetheless have important theoretical significance in terms of the mechanism of proton translocation accompanying respiration.

By incorporating a spectrometer capable of taking complete spectra in several milliseconds into a microcomputer-controlled system for studying the potentiometric characteristics of cytochromes, Dr. Hendler's group has found that mitochondrial cytochrome c_1 is actually two components, each of which transfers two electrons, rather than a single component which transfers one electron. With this new rapid scan facility, study of the kinetics of the oxidant-induced reduction of cytochrome b (originally observed by Chance and co-workers in 1952)

suggests that only cytochrome b_{566} is involved and not both cytochrome b_{562} and b_{566} as previously thought.

(6) Membrane Movements in Endocytosis: Last year, Dr. Bowers reported that Acanthamoeba has an internal mechanism for distinguishing between digestible and indigestible particles. The initial observation was that both latex beads and baker's yeast are readily phagocytosed by amebas, but only the yeasts are retained within the cells on subsequent phagocytic stimuli. Appropriate controls have now established that the presence of particles in the medium accelerates the exocytosis of latex beads, suggesting that endo- and exocytosis are coupled in some way. The working hypothesis is that the quality of digestibility of the yeast, rather than some surface or physical property, accounts for the difference in exocytic rates. By appropriate exchange experiments it was established that a phagocytic stimulus from either beads or yeast causes exocytosis of beads, but not of yeast. Furthermore, Dr. Bowers has shown that yeast rendered less readily digestible by extensive cross-linking with glutaraldehyde are exocytosed more readily than unfixed yeast. Since previous work in this laboratory has shown that bead phagosomes do acquire hydrolytic enzymes, the data so far support the idea that there are one or more metabolic events associated with digestion that override the external stimulus to exocytosis.

In order to examine the lysosomal system and to begin to document the fusions and interchange internal membranes related to the phagosome membrane/plasma membrane recycling in Acanthamoeba, Dr. Bowers' group has followed the release of lysosomal hydrolyses into the external medium. This release occurs at a low but detectable rate for several enzymes. The rates of secretion of five enzymes were characterized under three conditions: pinocytosis, phagocytosis of yeast, and phagocytosis of beads. In all cases the rate of secretion was linear with time, but secretion rates were not identical. (Control experiments showed that the secretion is dependent on an active process related to endocytosis.)

In pinocytosing cells acid phosphatase, β-glucosidase and α-galactosidase were secreted at a rate of 3% of the total activity per hour. Two of the five enzymes, N-acetylglucosaminidase and esterase were secreted at a rate of 14% per hour.

Results were different for phagocytosing cells. After saturation of cells with yeast, all enzymes were secreted at a rate of about 11% of the total activity per hour. On the other hand, after saturation of cells with beads secretion of acid phosphatase and β-glucosidase remained at about 3%, N-acetylglucosaminidase and esterase dropped from 14% to 3% and α-galactosidase increased to 15%. From these results, it appears that there are at least three distinct patterns of hydrolase release: one pattern is demonstrated by acidphosphatase and β-glucosidase, which are stimulated by the ingestion of yeast but unaffected by the ingestion of latex beads; a second pattern demonstrated by N-acetylglucosaminidase and esterase activities, in which hydrolase secretion is inhibited by the ingestion of both yeast and beads, with beads being the more effective inhibitor; the third pattern, exemplified by α-galactosidase, which is stimulated by ingestion of both yeast and latex beads. These observations are tentatively interpreted to indicate there may be subpopulations of lysosomes in

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00401-16 LCB

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Potentiometric studies of respiratory components of E. coli and mitochondria

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI's:	Richard W. Hendler	Head, Sec. on Membrane Enzymology	LCB NHBLI
	K.V.S. Reddy	Visiting Fellow	LCB NHLBI
Other:	Richard I. Shrager	Mathematician	LSMM DCRT
	David Songco	Computer Engineer	CSL DCRT

COOPERATING UNITS (if any)

Laboratory of Statistical and Mathematical Methodology, DCRT
Laboratory of Chemical Physics, NIAMD

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Section on Membrane Enzymology

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The enzymatic nature of the process for releasing acid when E. coli membranes are oxidized through a voltage range of 315 to 340 mV is indicated by the necessesity of providing E. coli protein, the loss of activity accompanying boiling of the protein fraction and the fact that mitochondrial protein has a different degree of activity in the system. Potentiometric titrations of beef heart mitochondria indicate that cytochrome c_1 exists as two 2 electron transferring species rather than as a single 1 electron species as now believed. Rapid scan spectroscopy of the phenomenon of the oxidant-induced reduction of mitochondrial cytochrome b reveals that only cytochrome b-566 participates in the reaction and not b-562. This is in contrast to contemporary schemes which place both b-cytochromes in the Q-cycle loop.

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Objectives: To identify and characterize the components of the respiratory chains of E. coli and mitochondria in terms of their redox potentials, numbers of electrons passed, amounts, spectra and possibilities for energy related changes in their properties. The main purpose for acquiring this information is to try to understand the structure of the electron transport chain and the mechanism for transduction of energy, liberated by oxidation, into metabolically useful forms.

Methods used and major findings:

A. Acid production accompanying oxidation through the voltage range 315 to 340 mV.

Requirements for this oxidative generation of acid are a protein fraction from E. coli membranes, a soluble amine, and a quinone. The protein fraction could not be replaced by an equal amount of beef heart mitochondria. However, if the mitochondria were treated with deoxycholate, acid generation did occur at about 10% the amount per equivalent weight of E. coli protein. This points to a requirement for protein and for a specificity in terms of acid generation efficiency. Boiling the E. coli protein fractions in the presence of 0.04 mM Cu^{2++} reduced acid generation by 80%. Inhibition was reduced to 40% in 0.06 mM Cu^{++} and 15% in 0.08 mM Cu^{++}. Cu^{++} has been found to participate in the acid generation process and the development of the EPR signal at g=2.067 which arises during the oxidative acid generation. A definitive experiment to test the conclusion that the system generates acid by oxidizing water is to look for the generation of O_2 concomitant to the formation of acid. To this end a collaboration has been initiated with Dr. Linus Barnes of the National Bureau of Standards. Dr. Barnes has designed, ordered the parts and constructed apparatus to trap effluent gases swept through the reaction system by argon gas. The water in the cuvette will be enriched with $^{18}O_2$. The effluent gases will be passed over carbon in a furnace of controlled temperature to generate CO_2 which will be collected in a cold trap that can be sealed and removed from the system. Mass spectrometry will be used for quantification of ^{18}O content in the CO_2. This equipment is currently being tested at the Bureau of Standards.

B. Studies with beef heart mitochondria.

There is now more uncertainty about the composition and structure of the mitochondrial electron transport chain than there was a few years ago. It is not known whether the chain is linear or whether the b-cytochromes together with coenzyme Q form a cyclic loop to pump protons. A long standing mystery, namely an oxidant-induced reduction of cytochrome b is not satisfactorily explained by the known facts. Dr. Reddy, who joined the laboratory in November, 1981, is involved in a study designed to provide new information about these problems. The approaches and techniques previously developed here to study the spectral potentiometric properties and the make-up of the E. coli respiratory chain are being used. In addition, we have written computer software that has now integrated a rapid scan spectromteter into the system so that transient states of reduction of the cytochromes can be observed. Results obtained so far, show that cytochrome c_1 which is figured in all schemes as a single 1 electron component is really two 2 electron species. The values of the 2 mid-point potentials are sufficiently close that, in the absence of

a discriminating analyses, it appears that only a single species is present. In the oxidant-induced reduction of cytochrome b (actually b_{562} and b_{566}), the explanation involving the linear chain is that the redox potential of cytochrome b is regulated by an undefined member of the electron transport chain, X, which when oxidized, bestows on b a higher affinity for electrons leading then to its reduction. This new species is stabilized by antimycin. We have tested this hypothesis by performing potentiometric titrations of mitochondria in the presence and absence of antimycin. No shift in the redox potential of b has been observed. The explanation involving the chain with a Co-Q, cyt b cycle is that oxidation of QH_2 is necessary in order to form $QH^{\cdot}$, which is the reductant for cytochromes b_{562} and b_{566}. With intact mitochondria, the oxidant induced reduction of b is short lived (< 10 sec) and we have been able to observe and study the phenomenon by taking spectra every few tenths of a second following successive additions of electron donor, inhibitors and oxidant. The advantage of our system over any other that has been used is that we take complete spectra rather than observing ΔA between two fixed wavelengths. Our approach has shown that it is only cytochrome b_{566} which is transiently reduced by oxidation. Cytochrome b_{562} does not participate and therefore it is not arranged in a cyclic loop which, if it exists, must involve only the one species of cytochrome b.

Biomedical Significance: Cell viability and health depend on the maintenance of adequate electric membrane potentials and energy supplies. Ischemia and anoxia lead to irreversible cell and tissue damage. A basic understanding of the molecular processes by which cells maintain adequate energy and electric balances may lead to means for preventing excessive damage under conditions of borderline levels of oxygen supply.

Proposed course: E. coli membranes will be suspended in ^{18}O-enriched water and effluent gases will be trapped and analyzed for $^{18}O_2$ in order to test the conclusion that H_2O is the source of acid obtained when the voltage of the system is raised through the 315 to 340 mV range. We will try to recognize and characterize all of the components of the mitochondrial electron transport chain using the spectral-potentiometric techniques and mathematical analyses developed in this laboratory. The phenomenon of oxidant-induced reduction of cytochrome b will be studied with the rapid scan spectrometer which is now integrated into a computer-controlled system. A collaboration has been established with Dr. John Rieske, of Ohio State University, whose work was used by Dr. Peter Mitchell in the formulation of the coenzyme Q cycle, and with Dr. Barry Bunow a thermodynamacist at NIH, interested in bioenergetic phenomena, to examine the thermondynamic and practical implications of the Coenzyme Q cycle. Our discussions so far point to serious thermodynamic flaws in current formulations of this model for the arrangement of the electron transport chain.

Publications: Resolving the Difference Spectra and Transitions of Individually Titrating Components in a Mixture. R.I. Shrager and R.W. Hendler, Anal. Chem. 54, 1147 (1982).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00402-10 LCB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

DNA Synthesis in E. Coli

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Richard W. Hendler	Head, Section on Membrane Enzymology	LCB	NHLBI
	Raymond Scharff	Physiologist	LCB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Section on Membrane Enzymology

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	1.25	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The complex made by treating commercial, purified DNA polymerase I with factor "E" is the same as DNA-synthesizing complex found naturally insofar as its electrophoretic mobility in detergent and non-detergent gel systems, its elution volume on BioGel A-1.5m columns, its reactivity with native DNA and its stimulation by ATP. Factor E, retained by the membrane fraction after complex has been released by EDTA treatment, is released by sonication but it is still excluded from a BioGel A-50m column and is sedimentable at 100,000 xg. The E obtained after these procedures is freer from contaminating activities of DNase and polymerase, both of which are mostly non-sedimentable. DOC extraction of the EDTA-treated membrane produces a form of active E which is included in a BioGel A-1.5 m column. An ATP-Sepharose column will bind complex only if Mg-2+ (10 mM) is present. The bound complex is then eluted with ATP. As in all previous manipulations involving complex, recovery of activity is dependent on the presence of factor E.

PHS-6040
(Rev. 2-81)

Objectives: To isolate and characterize a natural DNA-synthesizing complex which contains DNA polymerase I and the rec BC enzyme. To isolate and characterize a factor, E, necessary for the formation and stability of this complex.

Background: We have described a DNA-synthesizing complex in E. coli which contains DNA polymerase I, is active with native DNA and is stimulated by ATP. A factor has been found ("E") which is required for the stability of the complex and which can reform complex after it has been dissociated by dilution. The crude factor can also form a complex from commercially purified DNA polymerase I.

Major Findings and Methods Used: The identity of complex formed by action of crude factor E on commercial preparations of DNA polymerase I has been established as the same as that of natural complex. This is based on the following equivalence of properties:

1. Electrophoretic mobility in both detergent and non-detergent polyacrylamide gel systems.
2. Enhanced activity toward native DNA as compared to the activity of free DNA polymerase I and stimulation of this activity by ATP.
3. Poor recovery (<5%) of free DNA polymerase I from BioGel A-1.5m columns, but good recovery (~70%) of both natural and de novo complex from the same columns.
4. Elution volume from BioGel A-1.5m corresponding to ~390,000 M.W. The M.W. for DNA polymerase I is 109,000.

Initial steps for the isolation of factor E have been taken. After EDTA treatment has released most of the complex from the membrane fraction, sonication of the membranes releases E activity in a form not sedimentable at 20,000 g and which is excluded from BioGel A-50m columns. This activity can be sedimented at 100,000 g leaving much of the contaminating DNase and polymerase activities in the supernate. The sonicated membranes which sedimented at 20,000 g are still a rich source of E activity which can be released in a form not sedimentable at 100,000 g by extraction with deoxycholate. E-activity in this extract is now included in a BioGel A-1.5m column. Pre-treatment of the EDTA-extracted membranes with DNase prior to DOC extraction produces a peak of E-activity in a BioGel A-1.5m column which is much freer from contaminating DNA polymerase activity.

The DNA-synthesizing complex can be removed from solution by an ATP-Sepharose column but Mg^{++} is essential for such binding. The bound complex can be eluted with ATP. As in all other manipulations involving complex, E is required during these steps.

Biomedical Significance: The replication and repair of DNA is of fundamental importance to the maintenance of cell viability and health. Although it is generally appreciated that complexes of enzymes carry out these vital processes, very little is known about the identity of such complexes. It is likely that certain illnesses will be found in which there is a defect in the formation or activity of DNA-synthesizing complexes.

Proposed Course: Attention will be centered on the isolation and characterization of factor E, because the purification of complex is impossible without providing this stabilizing factor. We have learned that active E can be released from the membrane by deoxycholate. We will pursue this lead in the hopes of purifying E.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00405-08 LCB

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Circular Dichroic (CD) Studies of Protein Denaturation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Frederick H. White, Jr. Research Chemist LCB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Cellular Biochemistry and Ultrastructure

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

From previous work (Z01 HL 00405-07 LCB) and present efforts, organic solvents (methanol, 2-chloroethanol, and trifluoroethanol, which are known helix-formers) have been shown to have no effect on producing structure within the reduced lysozyme monomer, but only a continued randomization. This observation supports the previously stated hypothesis that SS bond formation is essential for a major transition to native structure. In a continuing investigation of factors that influence the structural state of reduced lysozyme, N-acetyl glucosamine (NAG) has been found to alter the CD spectra of fully reduced lysozyme and its carboxymethyl (CM) derivative. Computer curve fitting studies indicate an increased helical content of 1-3% of the chain length, with a decrease in β structure of similar magnitude. The observed changes, together with preliminary equilibrium dialysis studies, suggest an interaction between NAG and a rudimentary binding site, not known to exist in the reduced protein. Interactions of NAG and other substrate analogues with reduced lysozyme are under continuing investigation.

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Objective: To examine conformational structures that exist under a variety of conditions, in the absence of tertiary structural cross links, as these structures may relate to the folding process.

Methods:

1. Original methods are being used for both partial and complete reduction of SS bonds in lysozyme in the presence as well as absence of denaturation (F. White, Biochemistry 21, 967 (1982)).

2. CD examination of the products under a variety of conditions is carried out with a Cary 60 Recording Spectropolarimeter and a Model 6001 CD Attachment. Processing of the data is accomplished by curve fitting with published sets of "basis spectra," using the "MLAB" system of the PDP-10 Computer. This process gives estimates of the percent of chain length as α-helix, β structure, and aperiodic structure.

3. Original methods are being used for enzyme activity assay and for titration of SH groups with 5,5'-dithio-bis-(2-nitrobenzoic acid) as well as with p-chloromercuribenzoic acid.

4. An original procedure for ion exchange chromatography, employing diethylaminoethyl Sephadex in 5 M urea, is being used for fractionation of partially reduced lysozyme, after carboxymethylation, into components at various reduction levels (F. White, Biochemistry 21, 967 (1982)).

5. A Beckman Amino Acid Analyzer, Model 120, is used to determine the content of S-carboxymethylcysteine of reduced carboxymethyl (CM) samples of lysozyme (after acid hydrolysis) as a check on reduction level.

6. Gel filtration chromatography, employing BioGel P-60, is being employed as a means of differentiating between monomeric and polymeric forms of reduced lysozyme under various solvent conditions.

7. Equilibrium dialysis has been employed to investigate the binding of N-acetyl-D-glucosamine (NAG) to reduced CM lysozyme, in comparison to native lysozyme. Tritiated NAG, from Amersham Corp. (specific activity = 3Ci/mmol) is being used for this purpose.

Major Findings: In a continuation of previous work (Z01 HL 00405-07 LCB) the investigation has proceeded along two lines: (1) studies on the effects of organic solvents on reduced lysozyme, and (2) studies on the effects of substrate analogues on the remaining structure after reduction of protein SS bonds. These are described below.

1. Solvent Effects: Earlier it was found that exposure of fully reduced lysozyme monomer, or its derivatized forms, to methanol resulted in changes of CD behavior that could be interpreted as increasing β structure and decreasing α-helix. Thus, the changes were away from the "native" condition, where more helix and less β structure would have been in evidence. This observation is contrary to literature reports, based mainly on subjective interpretation of CD curve shapes, according to which the reduced protein chain could be made

to gain back significant amounts of native secondary structure by addition of structure-forming solvents.

In a continuation of this study, gel filtration chromatography has been used as a means of investigating the extent to which polymerization might account for the observed CD changes on addition of structure-forming solvents. Thus, gel-filtration columns, equilibrated with various concentrations of methanol, 2-chloroethanol, or trifluoroethanol, have been employed in separation of polymers from the monomeric form of reduced lysozyme. The results indicated, in every case, that the content of polymer increases with concentration of organic solvent. CD studies on the separated components indicate that the changes in CD behavior prior to chromatography are entirely the result of polymerization. The monomer of reduced lysozyme does not gain structure, as evidenced by CD, but rather loses what little structure remains after reduction, as organic solvent concentration increases. Thus, these known "helix formers" have only a further denaturing effect on reduced lysozyme monomer.

These findings are consistent with the hypothesis that native tertiary cross linkages (which are, in the case of lysozyme, SS bonds) must form prior to a major development of native structure. This hypothesis is contrary to widely accepted theory that the fully reduced protein folds to the native conformation, as dictated initially by short and medium-range interactions, with SS bonds forming as a latter event to stabilize the already folded chain.

2. Substrate Analogue Effects: In a continuing study of factors that influence the structural state of reduced lysozyme, NAG has been found to alter the CD spectrum of fully reduced lysozyme and its CM derivative. Thus, the negative ellipticity is 25-30% greater from 210 to 215 nm in the presence of NAG, with a maximal difference of 1200 deg cm^2 $dmol^{-1}$. The most pronounced effect is seen with a ratio of NAG to protein of approximately 50/1 (mol/mol), in dilute phosphate of pH 3-3.5. Computer curve-fitting studies suggest an increased helical content in the presence of NAG (1-3% of the chain length), with a decrease in β structure of similar magnitude. No comparable effects of NAG have been observed on other CM reduced proteins (ribonuclease and chymotrypsin); nor has any change in CD behavior of reduced lysozyme been detected with D-glucosamine.

Preliminary equilibrium dialysis studies, with tritiated NAG binding to reduced CM lysozyme, indicate a ΔF° of approximately -3kCal/mol. Identical studies on native lysozyme indicate that ΔF° = -4kCal/mol.

These observations suggest an interaction between NAG and a rudimentary binding site, not known to exist in the reduced lysozyme chain. However, no lytic activity on cell walls could be detected for these samples. Interactions of NAG and other substrate analogues with reduced lysozyme are under continuing investigation, in view of a possible relevance to the process by which the reduced chain assumes native conformation.

Significance to Biomedical Research: This effort is aimed at a further elucidation of the factors involved in protein chain folding. Such information may contribute to understanding the relationship between primary structure and protein conformation, and ultimately make possible the reliable prediction of native conformation under a given set of experimental conditions, from knowledge only of the amino acid sequence. This capability would be basic to attack of problems relating biological functions of medical significance with specific

protein conformations. The extensive influence of SS bonds on determination of protein conformation, suggested by the results obtained thus far in this investigation, suggest that locations of cysteine residues within the primary structure in the unfolded protein chain would be critical in the determination of secondary structure.

Proposed Course of Project: Studies on the effects of structure-forming solvents are nearly concluded. However, the investigation of substrate analogue effects on the reduced chain are still in progress, and this effort will be emphasized as these effects relate to the extent and kind of secondary structural development that proceeds in their presence.

Publications:

1. F.H. White, Biochemistry 21, 967 (1982).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00409-12 LCB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Interaction of Actin and Myosin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Evan Eisenberg	Section Head, Cellular Physiology	LCB	NHLBI
Others:	Leonard Stein	Clinical Associate	LCB	NHLBI
	P. Boon Chock	Research Chemist	LB	NHLBI

COOPERATING UNITS (if any)

Laboratory of Biochemistry

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Cellular Physiology

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	3	0

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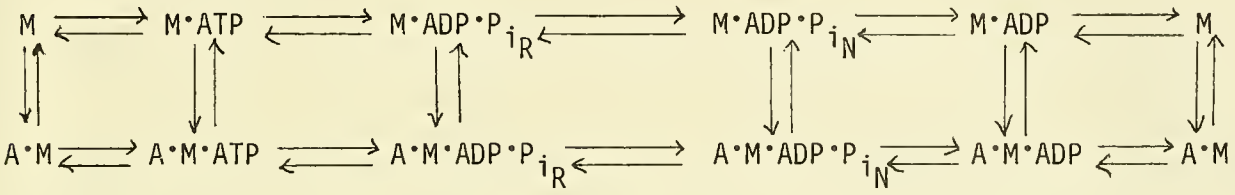
☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Steady-state and pre-steady state kinetic studies on the interaction of actin, myosin-subfragment-one (S-1) and ATP were carried out to better elucidate the mechanism of the actin-activated myosin ATPase. We have already shown that it is not required for myosin to detach from actin during each cycle of ATP hydrolysis. In the present study, we provide further evidence for the second major feature of our kinetic model -- that there is a special rate-limiting step, which preceeds Pi release and which occurs with the myosin either attached to or detached from actin. The existence of this special rate-limiting step can be proven by showing that the nucleotide on S-1 exists as bound ADP•P_i rather than ATP during steady-state ATP hydrolysis at high actin concentration. A preparation in which S-1 is covalently bound to actin has been used to demonstrate this point and indeed most of the nucleotide exists as bound ADP•P_i. This strongly suggests that the rate-limiting step in the ATPase cycle occurs after ATP hydrolysis but before Pi release. The importance of this step is that it controls the velocity of muscle contraction.

Objectives: The accepted mechanism for the contraction of muscle *in vivo* involves the interaction of actin and myosin filaments. However, the exact nature of the energy transduction mechanism by which the energy stored in the ATP molecule (relative to ADP and P_i) is converted to useful work is not well understood. By studying the biochemical interaction of actin and myosin *in vitro* we hope to gain insight into the *in vivo* energy transduction mechanism.

We have proposed the following model for the actin-activated myosin subfragment-1 (S-1) ATPase activity, based on pre-steady-state and steady-state studies of the binding of S-1 to actin in the presence of ATP, and the fact that no significant inhibition of the actin-activated ATPase by actin has ever been observed:



where M = myosin and A = actin. In this model, the S-1 molecule cycles between states weakly bound to actin ($M{\cdot}ATP$, $M{\cdot}ADP{\cdot}P_{i_R}$ and $M{\cdot}ADP{\cdot}P_{i_N}$) and states strongly bound to actin ($M{\cdot}ADP$ and M). There is a special rate-limiting step ($M{\cdot}ADP{\cdot}P_{i_R} \longrightarrow M{\cdot}ADP{\cdot}P_{i_N}$) in this model and this step can occur with S-1 either bound to or dissociated from actin.

During the past year, we have tested a key feature of this model: whether there is indeed a special rate-limiting step, which preceeds the release of P_i and which occurs at the same rate whether the S-1 is attached to, or detached from, actin.

Methods and Findings: The major reason for postulating the existence of a special rate-limiting step in our model is to explain the difference which occurs between the K_{app} for the ATPase activity (K_{ATPase}) and the binding constant of $S\text{-}1{\cdot}ATP$ and $S\text{-}1{\cdot}ADP{\cdot}P_i$ to actin ($K_{binding}$). Detailed studies carried out using both the A-1 and A-2 isoenzymes of myosin S-1 show that, at 15°, at very low ionic strength, K_{ATPase} is six- to eight-fold stronger than $K_{binding}$ for both $A\text{-}1{\cdot}S\text{-}1$ and $A\text{-}2{\cdot}S\text{-}1$. There is only one way these data can be explained by a model without the special rate-limiting transition from $M{\cdot}ADP{\cdot}P_{i_R}$ to $M{\cdot}ADP{\cdot}P_i$; that is to postulate that the hydrolysis step itself is rate-limiting when S-1 is attached to actin and the subsequent P_i release step is quite fast. However, if this were the case, the magnitude of the initial P_i burst should decrease greatly as the actin concentration is increased. To test whether this, in fact, occurs with $A\text{-}1{\cdot}S\text{-}1$, we previously measured the magnitude of the initial P_i burst as a function of actin concentration, using both fluorescence measurements, and direct measurements carried out with the three-syringe quench-flow apparatus. In both cases the results show that the magnitude of the initial P_i burst was much larger than would be predicted by a model without a special rate-limiting step which preceeds P_i release. However, it is difficult to make these measurements at high actin concentration and therefore, we also determined the magnitude of the initial P_i burst using

a new preparation of S-1 which is covalently cross-linked to F-actin. This preparation of S-1 has normal actin-activated ATPase activity and, in effect, is operating at infinite actin concentration. Using fluorescence measurements, our data strongly suggest that a large initial P_i burst occurs in this preparation. These data show that the ATP hydrolysis step is not the rate-limiting step in the model. Rather, there must be a special rate-limiting step in the model which follows the hydrolysis step but preceeds P_i release.

The finding that the ATP hydrolysis step occurs when S-1 is bound to actin is also important because it helps us to determine why O^{18} exchange, the exchange of O^{18} in H_2O with O^{18} in phosphate produced from ATP, is blocked by the presence of actin. In our work with Dr. John Sleep, a visiting scientist from England, we confirmed his original finding that O^{18} exchange did not occur at high actin concentration. Since this step is thought to be caused by the rapid equilibrium between M•ATP and M•ADP•P_i, and since our data on the ATP hydrolysis step show that a similar rapid equilibrium occurs between A•M•ATP and A•M•ADP•P_i, we can conclude that actin blocks O^{18} exchange by somehow preventing rotation of the phosphate at the active site rather than by preventing the rapid equilibrium between A•M•ATP and A•M•ADP•P_i.

In addition to our work with skeletal S-1, we also have carried on our experiments with smooth muscle S-1. These experiments show that smooth muscle S-1 follows the same basic kinetic mechanism as skeletal muscle S-1 in its actin-activated ATPase activity. However, the smooth muscle S-1 shows much less salt dependence in its binding to actin than the skeletal muscle S-1, and at physiologic salt concentration it binds considerably stronger to actin. This may be related to the higher force development by smooth muscle compared to skeletal muscle.

Significance to Biochemical Research: Understanding the mechanism of the actomyosin ATPase is central to gaining an understanding of muscle contraction as well as many other motile systems. This knowledge, in turn, may have important applications in the study of muscular dystrophy and heart disease.

Proposed Course of Research: During the next year, we plan to continue our investigation of the pre-steady state kinetic properties of skeletal muscle S-1 cross-linked to actin. We also plan to extend our cross-linking studies to smooth muscle S-1 and cardiac muscle S-1. The latter should be particularly useful since it has a rapid ATP hydrolysis step but a relatively slow actin-activated ATPase activity. Finally, we plan to extend our studies of S-1 binding to actin in the presence of ATP to studies with the two-headed HMM molecule. A new method of assaying binding with a radioimmune technique will be used in these studies.

Publications:

1. Hill, Terell L. and Eisenberg, Evan: Can Free Energy Transduction be Localized at Some Crucial Part of the Enzymatic Cycle. Quarterly Rev. Biophys. 14: 463-511, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 00411-07 LCB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Respiration-linked H-+ Ejection by Reconstituted E. Coli Succinoxidase Vesicles.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Richard W. Hendler	Section Head	LCB NHLBI
	Oruganti H. Setty	Visiting Associate	LCB NHLBI

COOPERATING UNITS (if any)

Laboratory of Statistical and Mathematical Methodology, DCRT

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Section on Membrane Enzymology

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Proton-liberation accompanying succinoxidase activity in vesicles reconstituted from soluble components resembles the process in uncoupled native membranes. Both amount to about 5% of the value in coupled membranes, they require an unperturbed membrane, and sufficient potassium to neutralize membrane potential. Assays for membrane potential and ΔpH using flow dialysis can lead to erroneous overestimations of the potential and underestimation of ΔpH when these values are below 100 mV.

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PHS-6040
(Rev. 2-81)

Objectives: Using as a starting point, membraneous succinoxidase vesicles, reconstituted from soluble fractions of the E. coli respiratory chain, we have sought to determine whether sufficient three-dimensional topography has been reformed to allow for asymmetric extrusion of protons during succinate oxidation. Our previous report described a succinoxidase-dependent acidification of the external medium but the H/O ratio was only about 5% of that of the original membrane and extruded protons did not return to the vesicular interior upon termination of the respiratory pulse. The object of the studies reported here was to determine whether acidification depended on membrane integrity or was merely a consequence of the enzymatic succinoxidase activity.

Methods Used and Major Findings: During the oxidation of succinate, two substrate level protons are produced. During the reduction of oxygen, an equivalent number of protons are consumed and so the oxidation of succinate per se should not lead to a net acidification. We have used two techniques to disrupt the integrity of the vesicle membrane in order to see if such treatment would dissociate succinoxidase activity from proton liberation. Treatment by brief sonication or low levels of deoxycholate each had only a slight effect on succinoxidase activity but accompanying acidification was more than 95% eliminated. These observations, together with the fact that lowering potassium concentration also dissociates oxidation from acidification, show that:

1.) Proton liberation is not a simple "scaler" process which accompanies succinate oxidation.

2.) The process of succinoxidase-dependent proton liberation requires a degree of membrane integrity which is lost by treatment with detergent or sonication.

3.) The process of proton liberation most likely generates a membrane potential which retards further proton liberation. Potassium is required to neutralize this potential.

All of the observations described for the reconstituted system apply equally to the native system that has been made leaky to potassium and protons by addition of valinomycin and FCCP. Our conclusion is that reconstitution forms a membrane with essentially correct orientation but without the permeability barriers necessary to conserve energy in the form of ion gradients. The system we are using may be useful in studying the factors which influence this property of membranes.

During the course of this work we observed some potentially serious artifacts which occur during the measurements of membrane potential and ΔpH by flow dialysis. These problems were studied and a report written and submitted for publication. The main findings are that with the usual electron donors and probes for membrane potential, oriented with a negative interior, the potential is underestimated, especially when its magnitude is <100 mV. Similarly, with probes for ΔpH oriented with an alkaline interior, the ΔpH tends to be underestimated at values <100 mV.

Biomedical Significance: Cell viability and health depend on the maintenance of adequate electric membrane potentials and energy supplies. Ischemia and anoxia lead to irreversible cell and tissue damage. A basic understanding of the molecular processes by which cells maintain adequate energy and electric balances may lead to means for preventing excessive damage under conditions of borderline levels of oxygen supply.

Proposed Course: The project has been completed.

Publications:

1. O.H. Setty, and R.W. Hendler. Interference of electron donors in the measurement of the proton gradient and membrane potential by flow dialysis. J. Biophys. Biochem. Methods, accepted for publication.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00413-06 LCB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

The Binding of the Subfragments of Myosin to Actin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: David L. Williams — Staff Fellow — LCB NHLBI
Lois E. Greene — Research Chemist — LCB NHLBI

Other: Evan Eisenberg — Section Head, Cellular Physiology — LCB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Cellular Physiology

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.58	3	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The effect of the regulatory proteins, troponin and tropomyosin, on the binding of myosin subfragment-one to actin was determined in the presence of different nucleotides. We found that in the presence of AMP-PNP, PPi, and ADP, tropomyosin alone confers slight positive cooperativity on the binding of S-1 to actin since there is a 3-fold increase in the affinity of S-1 for actin as the actin sites become saturated with S-1. The effect of tropomyosin on this binding is indistinguishable from that obtained with troponin·tropomyosin in the presence of Ca-2+. However, in the absence of Ca-2+, troponin·tropomyosin causes pronounced cooperativity in the binding of S-1 to actin, i.e. when the binding sites on actin are mostly unoccupied, troponin·tropomyosin strongly inhibits this binding, whereas when most of the sites are occupied, it strengthens the binding. These results indicate that troponin affects the binding of S-1 to regulated actin only in the absence of Ca-2+ and not in the presence of Ca-2+.

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Objectives: The equilibrium binding of S-1 to the tropomyosin·actin complex was compared to S-1 binding to the troponin·tropomyosin·actin complex to understand the separate roles of tropomyosin and troponin in the mechanism of muscle contraction. One of us (Greene) previously showed that S-1 binds with slight positive cooperativity to the troponin·tropomyosin·actin complex in the presence of Ca^{2+}, and with pronounced cooperativity in the absence of Ca^{2+}. We were interested in learning how tropomyosin alone affects this binding and, by comparison, how troponin affects S-1 binding to regulated actin. The experiments were performed with AMP·PNP and ADP, under the same conditions we used in studies to investigate the effect of troponin·tropomyosin on acto·S-1 binding. We also used PP_i, which may be particularly useful in relating in vitro studies to whole fiber studies since this ATP analog has no contaminating ATP or ADP and therefore will be unaffected by myokinase activity in the muscle.

Methods employed and major findings: The binding of S-1 to tropomyosin·actin and troponin·tropomyosin·actin in the presence of nucleotide was performed by mixing the actin complexes with varying concentrations of S-1 blocked at SH_1 with [^{14}C]-iodoacetamide. After centrifuging the acto·S-1 complex, the concentration of S-1 which remained in the supernatant was measured to determine the extent of binding. These studies were conducted in the presence and absence of Ca^{2+} since the Ca^{2+} concentration affects the cooperative response of troponin·tropomyosin.

Our results show that over a wide range of ionic strength (86-235 mM) tropomyosin confers slight cooperativity on the binding of S-1·AMP·PNP, S-1·PP_i and S-1·ADP to actin by strengthening this binding about 3-fold as the saturation of S-1 on actin increases from less than 2% to greater than 25%. The effect of tropomyosin on the binding of S-1·nucleotide (AMP·PNP, PP_i or ADP) to actin was found to be indistinguishable from that obtained with the troponin·tropomyosin complex in the presence of Ca^{2+}. As has been shown previously, at low saturation of the actin with S-1 the binding of S-1·nucleotide to troponin·tropomyosin·actin in the absence of Ca^{2+} is much weaker than is observed in the presence of Ca^{2+}, whereas at high saturation, the binding is strengthened about 3-fold. These results indicate that troponin has no effect on S-1 binding to regulated actin in the presence of Ca^{2+}. Only in the absence of Ca^{2+} does troponin weaken S-1 binding to regulated actin.

In another study examining the movement of tropomyosin on actin when S-1 binds to regulated actin, the troponin·tropomyosin complex is modified with a fluorescent probe. When S-1 binds to actin, there is a change in the fluorescence of this probe, which seems to correspond to the movement of the tropomyosin on the actin as it undergoes the transition from the weak binding state to the strong binding state. This change in fluorescence was examined when S-1 binds in the absence of nucleotide, in the presence of ADP, and the presence of PP_i. In all cases, the same percentage change in fluorescence was observed. Furthermore, even the binding of pPDM-modified S-1 (see Project No. Z01 HL 00510-01) to regulated actin induced the same percentage fluorescence change when examined in the absence of nucleotide or in the presence of ADP. However, in the presence of ATP, the binding of pPDM-modified S-1 to regulated actin had no effect on the fluorescence. These results indicate that there are at least two conformations of the tropomyosin on actin depending on the S-1 species bound to actin.

Significance to biomedical research: The interaction of actin with myosin in the presence and absence of tropomyosin and troponin·tropomyosin provides information on the basic mechanism of muscle contraction and its regulation. An understanding of the regulation of contraction is critical in the study of diseases of skeletal, cardiac, and vascular muscle.

Proposed course of research: The next step in this project is to investigate its relationship between the effects of tropomyosin on acto·S-1 binding with the effects on the acto·S-1 ATPase. At very low ratios of S-1 to actin, skeletal tropomyosin has an inhibitory effect on the acto·S-1 ATPase activity. At higher ratios of S-1 to actin, and at low ATP concentrations, skeletal tropomyosin has a potentiating effect on the acto·S-1 ATPase. However, if smooth muscle tropomyosin is added to skeletal acto·S-1, only potentiation of the ATPase is seen. Similarly, Acanthamoeba actin can activate the skeletal S-1 ATPase and skeletal tropomyosin only potentiates this hybrid acto·S-1 ATPase --- it does not inhibit it. Studies will be performed which compare the effects of smooth muscle tropomyosin with skeletal muscle tropomyosin on skeletal acto·S-1 binding, and compare the effects of skeletal muscle tropomyosin on skeletal muscle S-1 binding to skeletal muscle and Acanthamoeba actin. Parallel studies of the ATPases of these systems will also be conducted. These studies will tell us if the fraction of the tropomyosin·actin complex in the weak S-1 binding state is related to inhibition of the ATPase activity and conversely, whether the fraction in the strong S-1 binding state is related to potentiation.

The kinetics of the binding of S-1 to regulated actin will be studied by using light scattering in the stopped-flow apparatus. This will measure the amount of S-1 bound as a function of time. In addition, the kinetics of the transition of tropomyosin from the weak state to the strong state will be studied using fluorescence, after modifying the regulated actin with a fluorescence probe. These results will then be correlated with the equilibrium binding data to determine how these different aspects of the binding of S-1 to regulated actin fit together.

Publications:

1. Greene, L.E.: Comparison of the Equilibrium Binding of Heavy Meromyosin and Myosin to F-Actin in the Presence and Absence of the Troponin-Tropomyosin Complex. FEBS Lett. 139: 233, 1982.

2. Greene, L.E. and Eisenberg, E.: Interaction of actin and myosin in the presence and the absence of ATP. Methods in Enzymol. 85: 709-729, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00416-03 LCB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Studies on Plasma Components in Cystic Fibrosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Frederick H. White, Jr. Research Chemist LCB NHLBI

COOPERATING UNITS (if any)

Pediatric Metabolism Branch, NIAMDD
Division of Bacterial Products, Bureau of Biologics, Bethesda, MD

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Cellular Biochemistry and Ultrastructure

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.25	0.25	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The genetic defect of cystic fibrosis (CF) remains to be discovered, and its identification would likely produce improvements in the diagnosis and treatment of this disorder. In a continued search for this defect, the content of neuraminic acid derivatives (collectively termed the "sialic acid" content) in normal and CF sera have been analyzed by an HPLC method (R. Boykins and T.-Y. Liu, J. Biochem. and Biophys. Methods 2, 71 (1980)), not previously used for this purpose. New and as yet unidentified components have emerged from chromatography of the sialic acid fraction of CF sera, in addition to the N-acetyl and N-glycolyl derivatives of neuraminic acid that appear in the serum of normal individuals. Investigation will be continued to improve upon the method of separation, to identify the new components, and to assess their relevance to the genetic defect.

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Objectives: To isolate and examine plasma components, principally proteins and their ligands, from normal individuals and CF patients, in search of differences that may relate to the genetic defect of CF.

Methods:

1. The techniques of R. Schauer (Methods in Enzymol. 50, 64, 374 (1978)) are used for isolation of the sialic acid content from human blood plasma.

2. Established methods (G. Ashwell et al., Adv. Enzymol. 41, 99 (1974); J.H. Roe, J. Biol. Chem. 212, 335 (1955); L. Warren, J. Biol. Chem. 234, 1298 (1971)) have been employed in monitoring the content of sialic acid during the above isolation.

3. The method of R. Boykins and T.-Y. Liu (J. Biochem. and Biophys. Methods 2, 71 (1980)) was employed in the separation of neuraminic acid derivatives.

Major Findings: The term "sialic acid" is used here to include all derivatives of neuraminic acid found in nature. Certain differences in the sialic acid content of the protein previously under investigation (α_2-macroglobulin for which see Report No. Z01 HL 00416-02) have led to the question of what differences might also exist in the whole plasma, and an investigation was undertaken to elaborate on this issue.

First, it was necessary to find a means for the separation of the neuraminic acid derivatives in serum. Thus, the chromatographic procedure of Boykins and Liu was explored, with their cooperation, as a potential means of fractionating these components. Results thus far indicate that this procedure may be promising for differentiating between the various neuraminic acid derivatives, and this makes the first time that their method has been put to this use.

The major components of the sialic acid fraction of normal serum are N-acetyl and N-glycolyl neuraminic acids. The presence of these components is also obvious in CF serum. However, in the latter serum, there are two additional components that elute close to the glycolyl derivative. Thus, the latter elutes at 13.6 ($\pm$ .2) min, whereas a slightly larger peak elutes at 13.0 ($\pm$ .2) min, "shouldering" with the glycolyl peak. In addition, a smaller shoulder precedes both of these components, with an elution time of 12.0 ($\pm$ 0.2) min. These new components remain to be identified but may be additional neuraminic acid derivatives, suggested by the similarity in chromatographic behavior with the glycolyl derivative.

Significance to Biomedical Research: The genetic defect of CF remains unknown, and its elucidation could be significant in producing a more effective treatment, as well as diagnosis, of this disorder. Thus, any new product characteristic of CF, but not of normal individuals, could potentially shed light on the nature of the genetic defect.

Future Course: Further efforts will be made to elaborate on the newly discovered components in CF sera, to isolate and identify them, and to assess their relevance to the genetic defect.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00417-03 LCB

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

The Mechanism of Regulation of Muscle Contraction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Joseph Chalovich	Staff Fellow	LCB	NHLBI
Others:	Lois E. Greene	Research Chemist	LCB	NHLBI
	Evan Eisenberg	Section Head, Cellular Physiology	LCB	NHLBI
	Bernhard Brenner	Visiting Fellow		
	Mark Schoenberg	Research Chemist	LPB	
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COOPERATING UNITS (if any)

Laboratory of Biochemistry

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Cellular Physiology

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.08	7	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Vertebrate skeletal muscle relaxes when Ca-2+ is removed from troponin-tropomyosin a complex of proteins on actin. We previously showed, using single-head myosin subfragment-1 (S-1) that this inhibition results from a decreased rate of a step in the ATP hydrolysis cycle rather than from sterically blocking the binding of S-1 to actin. We now find that the two-headed fragment of myosin, heavy meromyosin behaves similarly. These studies suggest that myosin may be attached to actin in relaxed muscle. In fact, our stiffness measurements of muscle fibers are consistent with attached myosin in relaxed muscle at 5°C, 20 mM ionic strength. Therefore, even in muscle fibers, troponin-tropomyosin can cause relaxation without preventing the binding of myosin to actin. These studies have also been extended to smooth muscle. Chicken gizzard muscle is relaxed by dephosphorylation of myosin. We find that this dephosphorylation acts by blocking a step in the ATPase cycle rather than by blocking binding of the myosin to actin. Thus, in both smooth and skeletal muscle, relaxation can occur without preventing the binding of myosin to actin.

PHS-6040
(Rev. 2-81)

Objectives: To determine whether regulation of vertebrate skeletal muscle and vertebrate smooth muscle are regulated by controlling the binding of myosin to actin or by controlling the flux through one or more steps of the cycle of ATP hydrolysis.

Methods Employed and Major Findings: Binding of HMM (prepared from the myosins of rabbit skeletal muscle or chicken gizzard muscle) to actin or actin·troponin-tropomyosin was measured by rapidly sedimenting the acto·HMM complex in an air-driven ultracentrifuge. The amount of unbound HMM was determined by measuring the ATPase activity of the supernatant. Under identical conditions, the rate of ATP hydrolysis was measured by the rate of liberation of $[^{32}P]$-P_i from $[\gamma\text{-}^{32}P]$ATP or by the pH stat method.

For vertebrate skeletal HMM, the ATPase rate in the absence of Ca^{2+} is about 1/20 the rate in the presence of Ca^{2+} although the binding to actin·troponin-tropomyosin is only slightly weakened at 30 mM ionic strength (from 1.1×10^4 M^{-1} in Ca^{2+} to 8×10^3 M^{-1} in the absence of Ca^{2+}). This shows that, as we found for the single-headed fragment of myosin, S-1, the double-headed fragment, HMM, is also regulated by blocking a step in the ATPase cycle rather than by preventing its binding to actin. For vertebrate smooth muscle the ATPase rate of the non-phosphorylated HMM was about one hundreth that of the phosphorylated HMM although the binding is only weakened by about a factor of 4. Kinetic analysis of the ATPase data suggest that there is a large change in the V_{max} with only a small change in the Michaeles constant. This shows that dephosphorylation of smooth muscle HMM does not inhibit the acto·HMM ATPase rate by preventing the binding of HMM to actin but rather by blocking a step in the ATPase cycle.

Thus, although regulation of vertebrate skeletal muscle contraction is triggered by the binding of Ca^{2+} to troponin-tropomyosin associated with actin, and regulation of vertebrate smooth muscle occurs by phosphorylation of myosin, in both cases, the effect of regulatory modification is to control the flux through the cycle of ATP hydrolysis with little or no effect on the binding of HMM to actin.

To determine whether relaxation of vertebrate skeletal muscle fibers occurs without preventing myosin from binding to actin, we have measured the stiffness of skinned muscle fibers at 4°C and 20 mM ionic strength. Rabbit psoas muscle fibers were skinned by brief glycerol treatment and the stiffness was measured by rapidly stretching the muscle fiber 0.5% of its initial length in 100 μsec.

At 4°C, 20 mM ionic strength, we found no difference in the in vitro binding of S-1 to actin·troponin-tropomyosin in the presence of ATP in the absence and presence of Ca^{2+}. Under these same conditions, the glycerinated muscle fiber was well behaved, i.e. it was relaxed in the absence of Ca^{2+}, it contracted in the presence of Ca^{2+}, and it went into rigor in the absence of ATP. The relaxed fiber had about 1/3 the stiffness of the active fiber, suggesting that 1/3 of the crossbridge are attached in relaxed muscle under these conditions. Although the number of attached crossbridges in relaxed fibers is significant, no force is produced in the relaxed fiber, whereas 1 kg/cm^2 of force is produced upon activation. Therefore, even in intact muscle fibers, regulation by troponin-tropomyosin can occur without preventing the binding of myosin to actin.

Significance to Biomedical Research: The mechanism of regulation of muscle contraction is important in the study of disorders of skeletal muscle, card-

iac muscle and smooth muscle. The present work has developed a new way of thinking about the regulatory processes of muscle. We have shown that regulation occurs by controlling a step (or steps) in the ATP hydrolysis cycle. In the future, we hope to determine exactly which steps are affected; this may ultimately lead to the ability to modulate this process artificially.

Proposed Course of Research: (1) Presteady-state analysis of the kinetics of ATP hydrolysis will be done in the presence and absence of Ca^{2+} to determine if the hydrolysis step of ATP is modulated. (2) The effect of Ca^{2+} on the phosphate release step of ATP hydrolysis will be determined. The vanadate-ADP complex of myosin S-1 will be used as an analog of S-1·ADP·P_i in these studies. (3) The binding of skeletal HMM to actin will be studied in greater detail. In particular, we hope to develop a radioimmunoassay to increase the sensitivity of our binding assay. (4) We plan to continue the stiffness measurements of vertebrate muscle fibers. We plan to study the changes in stiffness induced by nucleotides other than ATP and to study the effect of ionic strength on the stiffness of relaxed muscle fibers. We also hope to extend our studies to work with myosin from molluscan muscle which is regulated by the binding of Ca^{2+} directly to myosin rather than to troponin-tropomyosin on actin.

Publications:

1. Hill, T.L., Eisenberg, E., and Chalovich, J.M.: Theoretical models for cooperative steady-state ATPase activity of myosin subfragment-1 on regulated actin. Biophys. J. 35: 99-112, 1981.

2. Chalovich, J.M., and Eisenberg, E.: Inhibition of actomyosin ATPase activity by troponin-tropomyosin without blocking the binding of myosin to actin. J. Biol. Chem. 257: 2432-2437, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 00418-02 LCB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Electrochemical potentials of protons in energy-transducing membranes.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI's:	R.W. Hendler	Section Head	LCB NHLBI
	O.H. Setty	Visiting Associate	LCB NHLBI
Other:	David Songco	Computer Engineer	CSL DCRT
	Richard I. Shrager	Mathematician	LSMM DCRT
	Walter S. Friauff	Electronic Engineer	BEIB DRS

COOPERATING UNITS (if any)

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Biomedical Engineering and Instrumentation Branch, DRS

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Section on Membrane Enzymology

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	1.25	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES X☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have designed and built a mini-electrode which measures salicylic acid concentration. Together with the mini-electrode which measures tetraphenyl phosphonium concentration (described last year), we can now compute membrane potential, ΔpH and protonmotive force. In addition to these two electrodes, our system contains a pH and an oxygen electrode which allows computation of rates of H-+ extrusion and O-2 uptake as well as the H-+-/O ratio in respiring energy transducing vesicles or cells. To follow all of these parameters, suitable computer software and electronic interfaces were developed. The computer accepts the voltage information and digitally filters out noise, corrects for delays in electrode responses, computes the complicated logarithmic functions to derive membrane potential and ΔpH and controls the outputs to a multiple recorder. The system has been tested for accuracy and is now being used to study E. coli cells and beef heart mitochondria.

PHS-6040
(Rev. 2-81)

Objectives: To develop a system that measures the instant states of membrane potential ($\Delta\Psi$), pH gradient (ΔpH), protonmotive force (PMF), rate of proton extrusion, rate of oxygen uptake, and H^+/O ratios in respiring, energy-transducing cellular or organelle suspensions.

Methods employed and major findings: We have been able to construct a mini solid-state membrane electrode which measures the concentration of salicylic acid. Because salicylic acid distributes itself in the intracellular and extracellular spaces according to the ΔpH between the compartments, it can be a probe for ΔpH. The response of the electrode to salicylic acid concentration is not strictly Nernstian. To deal with this problem equations which relate the slope of the electrode to concentration have been derived and incorporated into the software developed to operate the system. Together with the electrode designed to measure the concentration of the probe for membrane potential, tetraphenyl phosphonium (TPP^+) (described in last year's report), the system is now capable of measuring the $\Delta\Psi$, ΔpH, and PMF of respiring vesicles. Suitable software to accomplish this has been developed. The TPP^+ and salicylic acid have virtually instantaneous response to changes in concentration, but the pH and oxygen electrodes have relaxation times (τ) of 2 and 5 seconds respectively. True electrode reading (E) is related to the observed reading by:

$$E_{real} = E_{obs} + \tau \, (dE_{Obs}/dt)$$

The microcomputer converts observed readings for these electrodes to real voltages by this equation. In so doing any noise in the electrode is greatly exaggerated by taking the derivative (i.e. dE/dt). The real readings are then used to compute the rate of change of concentrations which involves a second differentiation and a further exaggeration of "noise". To allow these procedures to work, improvements in electronic circuitry and construction of a software digital filter were required. As a result we are able to correct the electrode reading for the delays in response and determine $d[H^+]/dt$, $-\, d\,[1/2\ O_2]/dt$, and H^+/O ratios. The entire system then gives continuous readings for $\Delta\Psi$, ΔpH, PMF, proton extrusion rate, oxygen uptake rate, and H^+/O ratio. All outputs have been independently checked for reliability using known changes of concentration, a precision voltage source, and by doing comparative analyses by the newer and older or standard techniques.

In initial trials of the new system with E. coli we have found that maximum values for $\Delta\Psi$, ΔpH, and PMF are attained almost instantly upon the addition of a pulse of oxygen to a suspension of cells in anaerobic medium. The amount of oxygen necessary to build this electrochemical potential is less than 1% of that found in an air-saturated solution. The maximum H^+/O ratio is reached in <2 seconds after the injection and its value appears to be about double that which we and others have found using conventional techniques.

Biomedical Significance: Cell viability and health depend on the maintenance of adequate electric membrane potentials and energy supplies. Ischemia and anoxia lead to irreversible cell and tissue damage. A basic understanding of the molecular processes by which cells maintain adequate energy and electric balances may lead to means for preventing excessive damage under conditions of borderline levels of oxygen supply.

Proposed course: We will use the newly developed apparatus to study dynamic relationships between the two components of protonmotive force, $\Delta\Psi$ and ΔpH, and of the H^+/O ratio and the dependence of these parameters on oxygen uptake, cell concentrations, and the presence of various ionophores. These studies will be carried out with E. coli and beef heart mitochondria.

Publications: none

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00419-02 LCB

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Structure-Function Relationships in Eukaryotic Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Blair Bowers	Research Biologist	LCB NHLBI
Other:	Enrico Cabib	Senior Research Chemist	LCB NHLBI
	Rowena Roberts	Staff Fellow	LCB NHLBI

COOPERATING UNITS (if any)

Laboratory of Biochemistry and Metabolism

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Section on Cellular Biochemistry and Ultrastructure

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.3	0.8	1.6

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Experiments on the cellular control of endocytosis and exocytosis in Acanthamoeba have shown that the cell has a defined capacity for uptake, shows no selectivity of uptake if the particle binds to the surface, but does distinguish internally between digestible and indigestible particles. Cells loaded to capacity with latex beads or yeast will replace the beads but not the yeast if given a second phagocytic stimulus by either beads or yeast. It appears that the quality of digestibility of the yeast, rather than surface or physical properties, is the significant difference between particles. Yeast rendered less digestible by extensive cross-linking are replaced by a second phagocytic stimulus. These results suggest there are one or more metabolic events associated with the digestive process that block an external stimulus to exocytosis.

PHS-6040
(Rev. 2-81)

Objectives: These studies, conducted on two different eukaryotic systems, are in each case directed toward understanding the control mechanisms of a cellular event that has a morphologically identifiable end point. In the first study, we are examining factors that limit or accelerate endo- and exocytosis in Acanthamoeba. These studies have particular relevance to two areas of current interest in the Laboratory, namely membrane recycling, which appears to be tied to endocytic events in Acanthamoeba, and the association of actin with membranes, since the endocytic event appears to require intermittent "recruitment" of actin to a particular membrane site. In the second study (in collaboration with E. Cabib, NIADDK) the objectives are to elucidate the way in which yeast chitin synthetase, an enzyme found generally on the plasma membrane, can be selectively activated at only one site on the membrane to cause a localized deposition of chitin.

Methods Employed: Transmission electron microscopy is being used for morphological studies of fixed and embedded cells and for examination of purified proteins by negative staining. Phase and fluorescence light microscopy are used to monitor living cells. Standard biochemical procedures are used for enzyme activity and protein measurements. Cytochemical staining of thin sections was performed using colloidal gold stabilized with appropriate ligands.

Major Findings:

1. Acanthamoeba Endocytosis: Last year, we reported that Acanthamoeba has an internal mechanism for distinguishing between digestible and indigestible particles. The initial observation was that both latex beads and baker's yeast are readily phagocytosed by amebas, but only the yeasts are retained within the cells on subsequent phagocytic stimuli. Appropriate controls have established that the presence of particles in the medium accelerates the exocytosis of latex beads, suggesting that endo- and exocytosis are coupled in some way. The working hypothesis is that the quality of digestibility of the yeast, rather than some surface or physical property, accounts for the difference in exocytic rates. By appropriate exchange experiments it was established that a phagocytic stimulus from either beads or yeast causes exocytosis of beads, but not yeast. That is, the external stimulus to phagocytosis (and the coupled exocytosis) is performed equally well by beads or yeast. Furthermore, we have shown that yeast rendered less readily digestible by extensive cross-linking with glutaraldehyde are exocytosed more readily than unfixed yeast. Since previous work in this laboratory has shown that bead phagosomes do acquire hydrolytic enzymes, the data so far support the hypothesis that there are one or more metabolic events associated with the digestive process that blocks the external stimulus to exocytosis.

2. Chitin Synthesis in Saccharomyces: In continuing studies on the morphogenetic controls of chitin synthesis in the yeast, Saccharomyces cerevisiae, we have examined seven, temperature-sensitive cell cycle mutants. Using a specific electron microscopic marker for chitin we have examined the localization of chitin synthesis in mutants grown at the restrictive temperature. Controls have shown that mutant cells at the permissive temperature show normal distribution of chitin and the parental strain shows normal distribution at the restrictive temperature, so that the aberrant depositions of

chitin are, in fact, due to the mutations. In six of the seven mutants examined, chitin synthetase was activated over the entire plasma membrane so that the entire wall displayed evidence of chitin deposition. This is in contrast to the normal localization of chitin only in the septal area. The seventh mutant, the only one in which the molecular defect has been identified (deficiency in thymidylate synthetase), shows normal deposition of chitin. The results so far indicate only that cellular control of chitin synthetase activation is a very delicately balanced phenomenon that can be altered by many mutations that interfere with the orderly progression of cell cycle events. In the course of this study, we have made new observations on the specific morphological lesions of some of the mutants, and have developed new procedures for reliable embedding of yeast with intact cell walls.

3. Acanthamoeba Proteins: In collaboration with J. Kuznicki and J.H. Collins the state of assembly of phosphorylated and dephosphorylated Acanthamoeba myosin II as a function of enzymatic activity was examined by electron microscopy. Preliminary results suggest that filaments of a critical size are necessary for enzymatic activity.

T. Olszewski, with S. Mockrin, has worked out conditions for observing by negative stain electron microscopy the growth of actin filaments from polymer "seeds."

Significance to Bio-Medical Research: Phagocytosis is a major mechanism of human defense against infection. This process can be profitably studied in the ameba where experimental conditions are simplified and where phagocytosis is much exaggerated. Certain yeasts are pathogenic in humans. Understanding the control of synthesis of chitin, an important wall component, may aid in developing better therapies for infections.

Proposed Course of the Project:

1. The Acanthamoeba project requires an improved method of data quantitation. At present experiments are analyzed by counting in the light microscope the number of particles per cell in at least 100 cells per time point. While accurate, this method is unreasonably time-consuming. We will test the feasibility of using a laser-activated cell-sorter for quantitating particle retention and loss. If this technique gives accurate results, we will pursue the analysis of mechanisms that control exocytosis by modifying latex beads in specific ways to test retention.

2. Using an in vitro system of isolated yeast plasma membranes we will attempt to localize newly synthesized chitin to determine whether vectorial transport of the completed polymer is maintained.

Publications:

1. E. Cabib, R. Roberts and B. Bowers. Synthesis of the yeast cell wall and its regulation. Ann. Rev. Biochem. 51: 1982 (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00501-09 LCB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Actin Polymerization

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Edward D. Korn	Chief	LCB	NHLBI
	Stephen L. Brenner	Staff Investigator	PSL	DCRT
Other:	Larry Tobacman	Clinical Associate	LCB	NHLBI
	Stephen Mockrin	Staff Fellow	LCB	NHLBI
	Altaf Lal	Visiting Fellow	LCB	NHLBI

COOPERATING UNITS (if any)

None

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.5	4.5	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Computer modelling of the polymerization of actin shows the size of the nucleus for polymerization to be 4 and that the rate of nucleation of G-actin containing bound Ca-2+ is slower than for G-actin containing bound Mg-2+. At steady state, complete exchange occurs between actin monomers and actin polymers in the presence of 0.5 mM Mg-2+. ATP hydrolysis is faster than actin exchange. The actin exchange is inhibited by substoichiometric concentrations of cytochalasins and when ADP is substituted for ATP. These data, and other data, may not be simply interpretable by any one model (such as treadmilling or exchange-diffusion) for equilibration. Covalently crosslinked actin dimer binds cytochalasin B weakly (K-D = 50 μM) relative to polymer and does not interact with profilin, which binds to monomer. The dimer polymerizes with no detectable lag implying that the dimer is the nucleus in this case. Hydrolysis of ATP is slower than polymerization of dimer. It is concluded that polymerization of dimer and hydrolysis of ATP are not coupled and that polymer-bound ATP is hydrolyzed subsequent to polymerization.

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Project Description:

Objectives: Actin, a major protein in the cytoskeleton of all eukaryotic cells is essential for maintenance of cell shape as well as for numerous motile activities. In these processes, the polymerization of actin is very important. Actin monomer (G-actin) is a protein of 42,000 daltons which polymerizes to long double-helical filaments (F-actin) in a process characterized by a slow phase leading to the formation of a small oligomer which serves as the nucleus for subsequent rapid elongation at both ends. The size of the nucleus and the magnitude of the nucleation and elongation rate constants were two subjects of investigation this year. The major approach was computer modelling of the polymerization kinetics.

Normally, polymerization of actin occurs in the presence of ATP and one molecule of actin-bound ATP is hydrolyzed for every molecule of G-actin converted to F-actin. Hydrolysis of ATP continues after steady state has been reached, when the concentrations of G-actin and F-actin are both constant. The mechanism of the hydrolysis of ATP and the consequences of this hydrolysis were also investigated. In part, this was done by comparing the rates of ATP hydrolysis and the rates of exchange of G-actin and F-actin at steady state under a variety of conditions.

Covalently crosslinked actin dimer has also been used to model the polymerization reaction. In this case, the coupling between polymerization and hydrolysis of ATP at each of the two nucleotide-binding sites was studied.

Methods Employed and Major Findings:

1. Nucleus Size: The rate of polymerization of actin is a function of the concentration of oligomer ends and the rates at which actin monomers associate and dissociate from these ends. In the simplest model this can be written:

$$dA_f/dt = (C_p^t)(k^+A_g^t - k^-) \qquad (1)$$

where A_f is the concentration of actin subunits in filaments, C_p^t is the concentration at time "t" of filament ends, A_g^t is the concentration of actin monomers at time "t" and k^+ and k^- are the association and dissociation rate constants for the addition or loss of actin monomers from the filament ends. C_p^t will depend on the rate of formation of nuclei which, in the simplest model can be written:

$$dC_p^t/dt = K_N^t(A_g)^{n-1}(k^{t+}A_g - k^-) \qquad (2)$$

where K_N is the pre-steady state equilibrium constant for the formation of oligomer of size "n-1" (where "n" is the size of the nucleus) and k^+ and k^- are the association and dissociation rate constants for the addition or loss of actin monomers from the oligomer of n-1 size. For further simplicity, it is assumed that the rate constants in (1) and (2) are the same, although they need not be. Finally, K_N can be evaluated as:

$$K_N = [A_{n-1}]/[A_g]^{n-1}. \qquad (3)$$

With these equations, one can ask the computer to choose a nucleus size "n" and a value for the product of $K_N(k^+)^2$ that will fit a given polymerization curve. If the course of polymerization is followed for a range of initial actin concentrations, all the polymerization curves should be fit using the same value for n and $K_N(k^+)^2$, if the equations are a close enough approximation of the actual polymerization events.

Experimentally, polymerization was followed by monitoring the 20-fold increase in fluorescence that occurs when actin labeled with a pyrenyl group on cysteine 374 is converted from monomeric G-actin to polymeric F-actin. Experiments were carried out over a 5-fold range of actin concentration in buffers containing either 1 mM $MgCl_2$, 0.1 M KCl and 0.1 M KCl plus 1 mM $MgCl_2$ and an excess of ATP. When the monomeric G-actin was first equilibrated with low concentrations of Mg^{2+}, the polymerization curves could be fit very well with a nucleus size of 4. It was not possible to fit all of the curves using a nucleus size of either 3 or 5.

When the monomeric G-actin contained Ca^{2+} as the actin-bound divalent cation, polymerization in 0.1 M KCl (although slower than with Mg^{2+}-G-actin) could still be fit by equations (1) and (2) and a nucleus size of 4. However, when Ca^{2+}-G-actin was polymerized in the presence of Mg^{2+} it was necessary to add an additional term, apparently to account for the exchange of actin-bound Ca^{2+} for actin-bound Mg^{2+}. Thus, equation (2) became:

$$dC_p^t/dt = K_N(A_g^t)^{n-1}(k^+A_g^t - k^-)(1-e^{kt})^3 \qquad (4)$$

where k is the observed rate constant for the exchange of Ca^{2+} by Mg^{2+}. Using this equation, the polymerization kinetics could again be fit uniquely with a nucleus size of 4 and a value for k of 0.45 s^{-1} at pH 7 and 0.66 s^{-1} at pH 8. These rate constants are similar in magnitude and in the effect of pH to the rate constants reported by Carl Frieden for the exchange of Ca^{2+} by Mg^{2+} on G-actin.

In addition to the nucleation reaction, filament ends capable of elongation might also be imagined to form as the result of fragmentation of existing filaments. Under the conditions used, where the initial actin concentration is far from the critical concentration, it has not been necessary to introduce a fragmentation term. It does become necessary to add a fragmentation term to equations (2) and (4), however, if polymerization is initiated at actin concentrations close to the critical concentration (in agreement with Albrecht Wegner).

2. Elongation Rate Constants: The above analysis gives the nucleus size "n" and the product of the equilibrium constant "K_N" and the elongation rate constant "k^2." To determine the elongation rate constant, one can add an excess of nuclei in the form of pre-polymerized actin. In the present study, this was done by adding an amount of covalently crosslinked actin oligomers (see later) sufficient to abolish completely the lag phase due to nucleation in the polymerization of Mg^{2+}-G-actin. Under these conditions, the Ca^{2+}-G-actin polymerized at essentially the same rate as Mg^{2+}-G-actin demonstrating that actin-bound Ca^{2+} has little effect on the elongation rates. The rate of nucleation of Ca^{2+}-G-actin, therefore, is slower than the rate of nucleation of Mg^{2+}-G-actin.

3. Events at Steady State: After polymerization of actin in the presence of ATP is complete, hydrolysis of ATP continues although the concentrations of G-

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actin and F-actin remain constant. To determine the extent to which the hydrolysis of ATP was coupled to the interconversion of G- and F-actins, ATP hydrolysis was measured by the rate of release of $^{32}P_i$ from [γ-^{32}P]ATP in parallel with measuring the rate of equilibration of G- and F-actins. The latter was done by adding a tracer concentration of pyrenyl-labeled G-actin to the steady-state solution (too little pyrenyl-G-actin to disturb the steady state) and monitoring the increase in fluorescence as the labeled G-actin and unlabeled F-actin became interconverted.

In 0.5 mM $MgCl_2$, the G-actin and F-actin became completely equilibrated over several hours. The ratio of moles of ATP hydrolyzed to moles of G-actin converted to F-actin was 4/1 over a 3-fold range of actin concentrations (11 to 45 μM). The kinetics of conversion of G-actin to F-actin are being curve-fit to several possible models for equilibration.

Incorporation of G-actin into filaments was greatly inhibited by concentrations of cytochalasin B and D that were very much less than the actin concentration, compatible with the effect being due to the capping of filament ends. When MgADP was substituted for MgATP the incorporation of G-actin into filaments was very greatly reduced although still inhibited by cytochalasins.

In the presence of 0.5 mM Mg^{2+} + 20 mM KCl, the kinetics of actin exchange and ATP hydrolysis were similar to Mg^{2+} alone but the exchange never reached more than about 80% completion. In 2 mM Ca^{2+}, with or without 20 mM KCl present, equilibration of G- and F-actins reached only about 75% but the ratio of ATP hydrolyzed to G-actin incorporated in filaments was 1/1.

In contrast to all these results, when polymerization was carried out by addition of KCl only there was very little if any exchange between G-actin and F-actin although the steady-state ATPase activity was similar to that in the presence of divalent cations.

It seems most likely that only exchange-diffusion occurs in KCl alone and in Mg^{2+} in the absence of ATP. But there may be no one simple mechanism (either treadmilling or exchange-diffusion) that will explain all the data obtained in Mg^{2+} and ATP.

4. Covalently Crosslinked Actin Dimer: The preparation of this dimer by reacting F-actin with the bifunctional reagent phenylenebismaleimide was described in last year's annual report. Its molecular weight has now been confirmed as 85,000 by sedimentation equilibrium. Additional studies have shown that the dimer has the properties neither of monomeric G-actin nor polymeric F-actin. It binds [^{3}H]-cytochalasin B (as shown by equilibrium dialysis experiments) with a K_D of 51 μM. This is weak binding compared to F-actin (K_D = 0.01 μM) but stronger than for G-actin which shows no binding at all. Dimer does not interact with profilin (a protein that forms a 1:1 molar complex with G-actin) as shown by the absence of any effect of profilin on the exchange of free ATP with the dimer-bound ATP, on the dimer-ATPase activity, or on the rate of polymerization of dimer. Profilin inhibits all these properties of monomeric actin. On the other hand, the fluorescence spectrum of NBD-labeled actin dimer is the same as that of NBD-monomer and NBD-dimer and shows the same two-fold increase in fluorescence upon polymerization as does NBD-monomer.

Whether monitored by the increase in light scattering or the increase in fluorescence of the NBD label, polymerization of covalently crosslinked actin dimer is induced by KCl. When $MgCl_2$ is used to initiate polymerization, there is a slight lag but this lag is eliminated by the exchange of the dimer-bound

Ca^{2+} by Mg^{2+}. Thus, as for the polymerization of monomer, Ca^{2+} seems to inhibit slightly the rate of polymerization. The dimer appears to be the size of the nucleus in this instance.

As reported last year, covalently crosslinked actin dimer contains 2 bound molecules of ATP. One of these is rapidly exchangeable with ATP in solution while the other is only very slowly exchangeable. It is, therefore, possible to prepare dimer with either or both sites labeled with [γ-^{32}P]ATP and follow the hydrolysis of each of the two sites independently during the polymerization of dimer. Labeling of the rapidly exchangeable site only is accomplished by allowing the exchange with [γ-^{32}P]ATP to continue only overnight; both sites are labeled if the exchange is allowed to proceed for several weeks; only the slowly exchangeable site remains labeled if dimer with both sites labeled is exchanged with non-radioactive ATP overnight.

The experimental data show that under conditions (either KCl or Mg^{2+}) in which polymerization is complete within 2-3 minutes, it requires 6-10 minutes for the ATP at the rapidly exchangeable site to be completely hydrolyzed and that hydrolysis of the ATP at the slowly exchangeable site is very much slower. Thus, much less than 1 mole of ATP is hydrolyzed for every mole of dimer that polymerizes. The filaments initially formed will contain almost 100% of one nucleotide site still labeled with ATP and about 66% of the other site still labeled with ATP. These filaments then will contain about 83% ATP and 17% ADP.

From these data, it can be deduced that for covalently crosslinked dimer (and by inference also for the polymerization of monomer) the ATP that is hydrolyzed during polymerization is bound to the polymer and not to the monomer. The hydrolysis of ATP at the rapidly exchangeable site after steady state has been reached in the polymerization process occurs at about the same rate as the hydrolysis of ATP by polymer formed from monomer. Thus, the lag in ATP hydrolysis during polymerization is probably due to a more rapid polymerization and not a slower hydrolysis of ATP.

Proposed Course of Research: A number of possibilities are under consideration: (1) estimation of the dissociation rate constant for polymerization; (2) kinetic analysis of the polymerization in the presence of ADP by computer modeling; (3) effects of AMP-PNP and regulatory proteins on steady-state ATP hydrolysis and actin exchange; (4) attempts to dissociate ATP hydrolysis from polymerization of native monomer to determine whether the ATP hydrolyzed during polymerization is monomer- or polymer-bound.

Publications:

1. Mockrin, S.C., and Korn, E.D.: Isolation and characterization of covalently crosslinked actin dimer. J. Biol. Chem. 256: 8228-8233, 1981.

2. Brenner, S.L., and Korn, E.D.: Stimulation of actin ATPase activity by cytochalasins provides evidence for a new species of monomeric actin. J. Biol. Chem. 256: 8663-8670, 1981.

3. Korn, E.D.: Actin polymerization and its regulation by proteins from nonmuscle cells. Physiol. Rev. 62: 672-737, 1982.

4. Tobacman, L.S., and Korn, E.D.: The regulation of actin polymerization and the inhibition of monomeric actin ATPase activity by Acanthamoeba profilin. J. Biol. Chem. 257: 4166-4170, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00503-10 LCB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Structure, Assembly and Function of Microtubules.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Martin Flavin	Section Head	LCB NHLBI
Other:	Katzutake Miyatake	Visiting Associate	LCB NHLBI
	Adavi Murthy	Visiting Fellow	LCB NHLBI

COOPERATING UNITS (if any)

None

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SECTION
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3	3	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In the past year our principle ongoing project to determine the function of tubulin modification by reversible enzymatic addition of C-terminal tyrosine has beem temporarilly suspended. In continuing last year's investigation of a new brain protein that aggregates tubulin, the observation that precipitation was accompanied by a stoichiometric burst of GTP hydrolysis (such as accompanies microtubule polymerization) supported the possibility that this protein might have a specific function, such as blocking indiscriminate assembly. This has now become less likely from the observations that a) the basic fraction from brain cytosol contains more than 1 tubulin aggregating protein and b) polylysine produces most of the same effects. To begin a study of the effects of protein phosphorylation on the functions of MAP-2 (the principle microtubule-associated protein of brain) we purified a non-specific brain protein phosphatase. With the latter + cAMP-dependent protein kinase we have prepared MAP-2 containing from 2 to 18 moles of phosphate per mole protein.

PHS-6040
(Rev. 2-81)

Objectives: Our objectives are to ascertain the biological functions of microtubule modification by tyrosinolation and phosphorylation.

Methods Employed: Biochemical and cytological procedures as indicated under Major Findings.

Major Findings:

1. Tubulin-Aggregating Proteins from Brain (K. Miyatake): A basic protein fraction from brain cytosol (i.e. which bound strongly to CM-cellulose at low pH) was found last year to cause tubulin aggregation. The aggregate was found to contain only 1 protein besides tubulin. This TBP (tubulin-binding protein) had a molecular weight of 52,500 in SDS-PAGE. Aggregates formed instanteneously at 0° or 37°, and contained about 1/2 mole TBP per mole tubulin when the former was added at a saturating concentration. By negative staining, electron microscopy revealed a structureless microprecipitate. TBP appeared to be a candidate to specifically sequester tubulin, thereby functioning either to inhibit indiscriminate polymerization, or to promote ordered nucleation by maintaining high local concentrations.

During the past year it was found that tubulin aggregation at 37° was accompanied by a burst of GTP hydrolysis in approximate stoichiometry with the moles of tubulin precipitated. This supported the possibility that TBP might have a specific function in brain, since a similar burst of hydrolysis occurs at the exchangeable GTP site on tubulin during polymerization into ordered structures.

However we have also found that more than 1 tubulin-aggregating protein is present in the basic fraction from brain. Besides the original TBP-1, a TBP-2 has been purified which elutes earlier from CM-cellulose, and has a lower molecular weight by SDS-PAGE analysis. The TBP-1 fractions also contained small amounts of 2 very low molecular weight components that sedimented in the presence of tubulin. Tubulin aggregation by TBP-1 was inhibited by 0.1 M NaCl, by TBP-2 by 0.2 M; no other functional differences have been observed.

The multiplicity of TBP proteins prompted us to do some comparative experiments with polycations previously shown to interact with tubulin. Among these DEAE-dextran had previously been reported to mimic brain MAPs in inducing polymerization into normal microtubules with concomitant hydrolysis of GTP (PNAS 74, 5372 (1977)). We confirmed this result, and found a burst of GTP hydrolysis also accompanied tubulin aggregation by protamine (in this case yielding microtubules helically wrapped with protofilaments), histone (yielding equivocal structures), and ribonuclease and polylysine (no ordered structures detectable by negative stain technique). Further experiments were done with polylysine (mol. wt. 21,000) since it gave an apparently structureless aggregate similar to the TBP proteins.

The extent of tubulin aggregation was proportional to the amount of polylysine added, as measured by turbidity or by the amount of protein that could be pelleted by centrifugation. At 10° or 32° turbidity was maximal at the earliest measureable time after polylysine addition (1/2 minute), and tubulin was completely precipitated at an equimolar concentration of polylysine. Tenth molar NaCl had no effect. Aggregation was not accompanied by hydrolysis of ATP under any conditions, or of GTP at 10°. At 32° there was a burst of GTP hydrolysis over a period of 5 to 10 minutes (at least 10 times longer than the period of turbidity development). Hydrolysis then

continued at a very low constant rate; the amount hydrolyzed per hour was 1/10 of that hydrolyzed in the initial phase. The magnitude of the burst did not parallel the turbidity, but was maximal at a polylysine concentration precipitating about half the tubulin. At all polylysine concentrations 0.1 M NaCl increased the amount of GTP hydrolyzed by 2 to 5 fold.

A few differences have been found in the characteristics of tubulin aggregation by polylysine as against TBP proteins: high Mg^{2+} increased GTP hydrolysis with TBP-1 and decreased it with polylysine; salt inhibited aggregation by TBPs but not by polylysine. However the preponderantly similar characteristics between tubulin aggregation by polylysine and by TBP proteins suggest that the later may not have a physiologically important function in relation to brain tubulin.

2. MAP_2 phosphorylation (A. Murthy): MAP_2 is the predominant protein (mol. wt. 300,000 by SDS-PAGE) in vertebrate brain that coassembles in vitro in constant proportion to tubulin, and promotes in vitro assembly of pure tubulin. Current evidence suggests MAP_2 may be abundant only in dendritic processes of non-primate vertebrate brain cells. This very restricted distribution suggests a specific role in nerve function, to which there is so far no clue. However MAP_2 occupies such a dominating position in protein phosphorylation in brain that investigation of it from this standpoint can be justified in its own right. Vallee has shown that at least 1/3 of all the cAMP-dependent protein kinase in brain is firmly bound to MAP_2; in fact so far only exposure to cAMP has allowed dissociation of native kinase. MAP_2 could function to locally sequester the kinase, which when released would phosphorylate something else. But the obvious starting point in this new project is the well known phosphorylation of MAP_2 itself.

Our initial projects have been: a) to purify a protein phosphatase active toward MAP_2; b) to prepare maximally phosphorylated and dephosphorylated MAP_2 and compare their assembly-promoting properties; c) to characterize the MAP_2 sites accesible to different kinases and phosphatases.

As phosphatase substrates we prepared ^{32}P-labeled histone and MAP_2. The latter was prepared by autophosphorylation of tubulin-2xP, purified by boiling followed by molecular sieve fractionation, and concentrated with XM100 filters. The product, in 25% yield, contained 10 to 12 moles phosphate per 300,000 g protein (the highest value reported before now had been 6). Various commercially available alkaline and acid phosphatases (i.e. membrane-derived glycoproteins not primarilly active on phosphoproteins) were found to rapidly digest MAP_2 even in the presence of all available protease inhibitors (tubulin was more protease-resistant and the beta chain, or more likely a tau protein coincident in SDS-PAGE, appeared to be dephosphorylated by some of these enzymes).

In crude brain supernatant fluphenazine partially inhibited dephosphorylation of MAP_2. Calcineurin has recently been identified as a calmodulin-dependent protein phosphatase. Since calcineurin and MAP_2 are both at least 20 times more abundant in brain than in other tissues, we are currently investigating whether this so far very specific phosphatase acts on MAP_2.

Meantime, we have purified the non-specific, alcohol-stable protein phosphatase catalytic subunit from brain. By modification of published procedures it has been purified 600 fold in 22% yield. The following species of MAP_2 were then prepared and purified: a) unmodified, from tubulin-2xP; b) maximally phosphorylated, by autophosphorylation of 2xP, to contain 10

newly introduced phosphate residues; c) b treated with purified phosphatase, removing 85% of the P^{32}. Kinase was removed from (b) by the heat step, and phosphatase was removed from (c) by elution from BioGel A1.5m. Aliquots of each of these were then maximally phosphorylated with added purified cAMP kinase catalytic subunit (the enzyme from beef heart muscle, which has been shown to be identical with that complexed with brain MAP_2). Both (a) and (c) could accept 10 moles/mole, and PAGE analysis showed all the P^{32} to be in MAP_2. This suggested than MAP_2 as isolated from tubulin-2xP was completely dephosphorylated, and that no further sites had been exposed by phosphatase treatment. But (b) could also accept 8 more phosphates for a total of 18. At this point it appears that a different set of sites may be exposed in MAP_2 that has been separated from tubulin and/or heated to 100° C.

Tubulin assembly was promoted by (b) to a normal extent but at a much reduced rate. However (c) was incompetent, indicating that it was not just reconstituted (a), but had been altered in another unidentified way.

3. Miscellaneous projects: Dr. N. Kumar extended last year's studies of tubulin which has undergone phase-transition insertion into the lipid bilayer of neutral liposomes. In the presence of mM Ca^{2+} (but not Mg^{2+}) these liposomes underwent tubulin-dependent fusion into larger vesicles, without loss of internal aqueous contents, providing a possible model for Ca^{2+}-induced exocytosis. Further results relating specifically to the tubulin aspect of this project were that: MAPs markedly inhibited tubulin insertion; maximally tyrosinolated and detyrosinolated tubulins were inserted indiscriminately; inserted tubulin could bind colchicine and MAP_2, and was as accesible as free tubulin to tyrosinolating and detyrosinolating enzymes.

Dr. K. Miyatake devised a more convenient procedure for providing tubulin-tyrosine ligase in a paritally purified but stable form. Purification was 300 fold in 16% yield, and involved ion-exchange, phenylsepharose and Affi-Gel blue columns.

MAP_1 is a protein of slightly higher molecular weight than MAP_2 and co-assembles in comparable amounts during in vitro cycles of brain microtubule polymerization. Although it was believed at one time to be a dynein-like ATPase, this expectation has not been realized, and it has since been almost completely neglected. Recent reports that it is antigenically related to the 200 kilodalton HeLa MAP, and to red blood cell ankyrin, raise the possibility that it might be a bridging element between microtubules and the plasma membrane. Dr. K. Miyatake is currently undertaking to purify native MAP_1 away from MAP_2 by the use of MAP_2 antibodies or by various column procedures such as chromatofocusing or elution from DNA-sepharose. The MAP_1 + MAP_2 fraction can probably be isolated directly from brain extract by salt elution of taxol-induced microtubules.

Proposed course of project:

1. Phosphorylation of microtubule proteins: We will search for a brain protein phosphatase specific for MAP_2, and continue to investiage how calmodulin affects (de)phosphorylation of the latter, and whether its inhibition of MAPs-induced assembly is mediated by phosphorylation.

With maximally and minimally phosphorylated MAP_2 we will compare: assembly and treadmill kinetics; microtubule affinity for secretory granules, actin etc.; MAP_2 affinity for taxol microtubules. If the same kinase recog-

nizes different sites before and after MAP_2 has been heated, or perhaps in any case, we will become involved in effects of phosphorylation, limited to specific sites or clusters of sites, on MAP_2 function, and in the capacity of proteolytic fragments to serve as kinase or phosphatase substrates.

2. Tubulin tyrosinolation: The status of this project can best be conveyed by a series of paradoxical, or at least puzzling, results. Tubulin occurs in 3 species: with and without tyrosine, and a third "non-substrate" moiety which can not be enzymatically tyrosinolated even after all pre-existing tyrosine has been removed with carboxypeptidase A. When protein synthesis is completely inhibited by antibiotics HeLa cells fix tyrosine only into tubulin, but all of it goes into the non-substrate species. How is this brought about, and what is different about this tyrosine and/or this tubulin? So much tubulin is tyrosinolated that it could only be occuring by turnover (the substrate tubulin from HeLa is always completely tyrosinolated, a unique property of these cells). Yet none of the tyrosine fixed in vivo can subsequently be chased out. Finally, why is this tyrosine introduced by both genomic and post-transitional paths?

We believe the first step should involve pulse-chase experiments in HeLa with careful analyses of the specific radioactivity of the pool of free tyrosine. To this end, during the past year we have equipped the laboratory with facilities for cell culture and for hplc micro-amino acid analysis. The latter will also be applied to direct determination of tyrosine released from tubulin purified from HeLa; sequential uses of CPA and ligase can reveal how much of the non-substrate tubulin has C-terminal tyrosine.

The focal point is, what is "non-substrate" tubulin? Isolation of this species might be achieved with monoclonal antibodies specific for tubulin with tyrosine (which may become commercially available this year), or by introducing C-terminal dopa instead of tyrosine (the use of dopa obviously is also a vehicle to distinguish coded from modified protein).

Following are some related projects. Leishmania now provide a unicellular organism to study invertebrate and axonemal tyrosinolation. Culture capability will allow reinvestigation of tubulin changes during the HeLa cell cycle, and during neuroblastoma differentiation. Important differences in the properties of tyrosinolated and detyrosinolated tubulin which remain to be confirmed are: microtubule treadmilling rates, nucleation/elongation competence, and ultrastructure of taxol-induced polymers. Further questions are: is it only detyrosinolated subunits that shut down tubulin synthesis; what is the tyrosine catabolite that accumulates when leukocytes are stimulated with a chemoattractant; is there a detyrosinolating enzyme specific for non-substrate tubulin?

3. Miscellaneous: Nothing is known about MAP_1 except that it can promote tubulin assembly. In this respect we will determine whether it competes with MAP_2 or taxol, whether it can saturate tubulin, can form rings at 0°, has projection domains which appear as microtubule arms (and if so with what periodicity). The structure of native MAP_1 is completely unknown. If MAP_1 is a bridging element between membranes and microtubules, a search for things that bind to it should lead to other elements in the chain.

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2. Kumar, N. and Flavin, M.: Preferential action of a brain detyrosinolating carboxypeptidase on polymerized tubulin. J. Biol. Chem. 256, 7678-7686, 1981.

3. Nath, J., Flavin, M. and Schiffman, E.: Stimulation of tubulin tyrosinolation in rabbit leukocytes evoked by the chemoattractant formyl-methylionyl-leucyl-phenylalanine. J. Cell Biol. 91, 232-239, 1981.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00506-07 LCB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Acanthamoeba Myosins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

Dr. Klee is a member of the Laboratory of Biochemistry, NCI

LAB/BRANCH
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SECTION
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National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.5	6.5	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Acanthamoeba Myosin I heavy chain kinase has been purified. It is a monomeric protein of molecular weight about 105,000 which phosphorylates the heavy chains of both myosins IA and IB to a maximal extent of 1 phosphate/heavy chain and has no activity toward myosin II heavy chain. The $K_{binding}$ of F-actin is the same for the enzymatically active phosphorylated myosin I and the enzymatically inactive unphosphorylated myosin I and is the same as the K_{ATPase} for the active enzyme (1 μM F-actin). The enzymatically more active, dephosphorylated form of Acanthamoeba myosin II has a higher K-ATPase for F-actin than does the much less active phosphorylated form of the enzyme so that its greater enzymatic activity is also attributable to its greater V-max. The dephosphorylated myosin II does have a lower K-binding for F-actin than does the phosphorylated form of the enzyme. The dephosphorylated enzyme also forms larger, more stable (to KCl and MgATP) bipolar filaments than does the phosphorylated enzyme. Acanthamoeba calmodulin has been purified to homogeneity.

225

PHS-6040
(Rev. 2-81)

Project Description

Objectives: As reported in previous annual reports, Acanthamoeba castellanii contains three myosin isoenzymes. Myosin IA contains one heavy chain of molecular weight 130,000 and light chains of molecular weights 17,000 and 14,000; myosin IB contains polypeptides of 125,000, 27,000 and 14,000, myosin II contains two heavy chains of molecular weight 185,000 and two pairs of light chains of about 17,500 and 17,000. Myosins IA and IB exhibit actin-activated MgATPase activity only when the heavy chains are phosphorylated while myosin II exhibits actin-activated MgATPase activity only when its heavy chains are dephospnorylated. Each heavy chain of myosin II was shown to contain three phosphorylation sites while the heavy chains of myosins IA and IB seem to contain only one phosphorylation site. The general problem approached this year was that of understanding how the state of phosphorylation of the three myosin isoenzymes regulates their interaction with actin.

For the unique enzymes myosins IA and IB, the primary problem still is the difficulty in preparing sufficient quantities of the enzymes to allow the necessary studies. One of the goals this year was to establish a more reliable procedure that could be used routinely. The myosin I heavy chain kinase has previously been available only in crude form and, therefore another goal was the preparation of a highly purified kinase suitable for studying the phosphorylation reaction and for preparing, as and when desired, phosphorylated myosins IA and IB from the completely unphosphorylated forms that are isolated. With the availability of adequate quantities of unphosphorylated and phosphorylated myosins IA and IB it was then hoped to begin the characterization of their interaction with F-actin and possible self-associations.

Similar objectives were planned for the study of myosin II. In this case, the myosin is relatively easily prepared in rather large quantities but the heavy chain kinase is very unstable. Also, the phosphorylated, rather than the dephosphorylated form, of the enzyme is isolated and so it was desired to attempt to locate and purify a myosin II heavy chain phosphatase from the ameba. Mostly, the objective this year was to study the interaction of phosphorylated and dephosphorylated myosin II with F-actin and to compare the abilities of each to form filaments.

Finally, in most non-muscle cells the myosin kinases require Ca^{2+}/calmodulin for activity and are themselves phosphorylated by the action of cAMP-dependent protein kinases. Recent evidence suggests that some phosphatases may be regulated by Ca^{2+}/calmodulin. All previous efforts to show an effect of mammalian calmodulin on the ameba enzymes were unsuccessful but the possibility remained that the Acanthamoeba calmodulin might be different. Therefore, it was decided to attempt to purify calmodulin from Acanthamoeba although there was one report in the literature that Acanthamoeba does not contain calmodulin.

Methods Employed and Findings:

1. Purification of Myosin I Heavy Chain Kinase: This enzyme has now been prepared in about 95% purity as judged by Coomassie Blue staining of SDS gel electrophoretograms. Its purification is monitored by its ability to activate the actomyosin ATPase activity of myosins IA and IB. The procedure involves KCl gradient chromatography of the unadsorbed protein fraction from the DE-52

column used to purify myosins IA and IB. The major peak of activity eluting at about 0.15 M KCl is then chromatographed on a red-dye column eluted with a KCl gradient. Most of the activity is tightly bound, eluting at 0.9 M KCl. This fraction is then chromatographed on a histone-Sepharose affinity column eluted with MgATP. Essentially homogenous kinase is eluted with 20 mM MgATP.

About 200-400 µg of kinase is obtained from about 1200 g of amebas (30 g of protein). The material shows essentially one band, of about 105,000 daltons, on SDS-gel electrophoresis. The enzymatic activity chromatographs on gel filtration columns as a protein with a Stoke's radius of 4.1 nm which is compatible with a globular protein of 110,000 and an axial ratio of 1.3/1. These data suggest the active enzyme is a monomer and support the assumption that the major band seen on electrophoretic gels is the kinase.

The kinase catalyzes the phosphorylation of the heavy chains of Acanthamoeba myosins IA and IB to a maximum extent of 1 mol of phosphate/mol of heavy chain. It has a specific activity under the assay conditions used of about 200-300 nmol/min/mg. Phosphorylation of myosins IA and IB fully activates their actomyosin ATPase activity without the need for any other effectors.

The kinase has no activity with Acanthamoeba myosin II as substrate, has a specific activity with casein of 50 nmol/min/mg, with histone 2A (its best substrate) of 700-800 nmol/min/mg, and has no activity with phosvitin as substrate. Activity is maximally inhibited to the extent of 40% by 10-30% glycerol and is inhibited completely by 200 mM KCl. Myosin I heavy chain kinase has no demonstrable requirement either for cAMP or Ca^{2+}/calmodulin although the possibility must be considered that a regulatory site on the protein has been lost during the isolation of the enzyme.

2. Acanthamoeba Myosins IA and IB: For the first time since their discovery more than 10 years ago, these two enzymes can routinely be prepared in about 90% homogeneity with no degradation of their polypeptide subunits and with sufficient stability to allow their study. This has been accomplished by a number of major modifications of the procedures developed in this laboratory and described in previous annual reports. It still requires about one week, however, to prepare about 5 mg of each of the two isoenzymes and this small amount does not allow many structural studies before another preparation is required. As isolated, myosins IA and IB have essentially no actin-activated MgATPase activity but are fully activated by phosphorylation by the myosin I heavy chain kinase, as just described.

To understand the basis of the regulation of actomyosin ATPase activity by phosphorylation, the concentration of F-actin required for 50% binding under assay conditions have been compared. The assay conditions are: 2 mM ATP, 3 mM $MgCl_2$, 0.5-1 mg/ml bovine serum albumin, 15 mM imidazole, 2 mM EGTA, pH 7.5. The albumin is present to prevent inactivation during the binding studies and has no effect on enzymatic activity. Binding is studied by the co-sedimentation of the myosin with F-actin in the Airfuge, the myosin concentration in the supernatant being measured by its K^+, EDTA-ATPase activity. The reciprocal plot of the fraction of myosin IB bound to F-actin as a function of F-actin concentration gives a $K_{binding}$ of about 1 µM for phosphorylated and dephosphorylated myosin IB. The reciprocal plot of the actin-activated ATPase activity as a function of F-actin concentration gives a K_{ATPase} with an identical value of 1 µM for phosphorylated myosin IB. The very low activity of the unphosphorylated myosin IB makes a K_{ATPase} impossible to determine. Thus, phosphorylation seems to have no effect on the binding of

myosin IB to F-actin and, therefore, the effect of phosphorylation on enzymatic activity must be entirely an effect on V_{max}. Partial data for myosin IA indicate that it will behave identically to myosin IB. It is interesting that $K_{binding}$ of 1 μM is much tighter than the value for F-actin and muscle myosin subfragment-1 (is about 20 μM according to Greene and Eisenberg) since subfragment-1 is also a globular single-headed molecule.

Physical studies have firmly established that myosins IA and IB are both monomeric molecules. By sedimentation-equilibrium analysis in 0.5 M KCl both enzymes have molecular weights of about 180,000 and by gel filtration on Bio-Gel A1.5m both proteins have a Stoke's radius of about 5 nm. These data are consistent with their being globular molecules consisting of a single chain of 130,000, 17,000 and 14,000 (myosin IA) and 125,000, 27,000 and 14,000 (myosin IB). The proteins have different circular dichroism spectra but each contains very little α-helix, only about 5%, also consistent with a highly globular structure. This globular protein has been visualized by rotary shadowing electron microscopy and has a size very similar to S-1, as would be expected.

The structural considerations make it difficult to imagine that either myosin IA or IB could form filaments and it has, in fact, been impossible to demonstrate filament formation or even formation of any oligomers in buffers containing from 2 to 6 mM $MgCl_2$, no KCl, 10 mM Tris, pH 7.5 and 5% glycerol. Under all conditions tried, sedimentation-equilibrium analysis shows only monomer to be present.

3. Acanthamoeba Myosin II: We had reported in last year's annual report that myosin II, as isolated from the amebas, by the procedure of Collins and Korn, contains about 1.2-1.5 mol of phosphate per mol of heavy chain and that this form of the enzyme was optimally active at a Mg^{2+} concentration of 5.5 mM, at which concentration fully phosphorylated myosin II (3 mol of phosphate per mol of heavy chain) had essentially no actin-activated MgATPase activity. We now have found that maximally dephosphorylated myosin II (less than 0.5 mol of phosphate per mol of heavy chain) has maximal actin-activated MgATPase activity at a lower concentration of Mg^{2+}, 4 to 4.5 mM and that the fully phosphorylated enzyme does show some activity at very high concentrations of Mg^{2+}, about 7 mM. At all concentrations of Mg^{2+}, however, the dephosphorylated enzyme is appreciably more active than the phosphorylated enzyme. Most of this year's efforts have been devoted to studying the effects of the state of phosphorylation of myosin II on its interaction with F-actin and on the self-association of myosin II molecules.

Double reciprocal plots of the actin-activated MgATPase activity of fully phosphorylated and maximally dephosphorylated myosin II as a function of F-actin concentration were linear. The kinetic constants obtained at 5 mM Mg^{2+} were: dephosphorylated myosin II, V_{max} = 3 s^{-1} and K_{ATPase} = 4 μM; phosphorylated myosin II, V_{max} = 0.2 s^{-1}, and K_{ATPase} = 1 μM. Thus, the V_{max} is about 15 times greater for the dephosphorylated enzyme while the K_{ATPase} is 4 times higher for the dephosphorylated enzyme. From these data one can conclude that the greater enzymatic activity of the dephosphorylated enzyme is a function of the turnover number of the catalytic site and not related to the concentration of F-actin required to activate the two forms of myosin II. In fact, the less active, phosphorylated form of myosin II is activated by a lower concentration of F-actin.

Binding studies were then carried out under conditions identical to those of the enzymatic assay. The fraction of the myosin II that sedimented in the

Airfuge with F-actin under conditions in which the myosin II did not sediment alone. Residual myosin II in the supernatant solutions was determined by measuring the soluble ATPase activity (and ^{32}P in the case of phosphorylated myosin II). Reciprocal plots of the fraction of myosin II bound as a function of F-actin concentration were linear for both dephosphorylated and phosphorylated myosin II and gave values of $K_{binding}$ of 0.3 and 1.6 μM, for the dephosphorylated and phosphorylated enzymes, respectively. Thus, the binding of dephosphorylated myosin II to F-actin has a lower apparent dissociation constant than does the binding of phosphorylated myosin II. This is opposite to the ratio of their K_{ATPase} values. It should be remembered that the K_{ATPase} is a kinetic constant and not a binding constant.

Thus, it appears that the dephosphorylated form of myosin II has a greater affinity for F-actin as well as a higher turnover number at the catalytic site than does the phosphorylated form of the enzyme. The binding data are somewhat complicated, however, by the fact that both forms of myosin II are probably filamentous under the conditions of the assay and the data are further complicated by the fact that the dephosphorylated form probably exists in larger filaments than the phosphorylated form. These binding data cannot then be directly taken as measures of the affinities of myosin II heads for F-actin which can only be accurately measured with a soluble form of myosin such as heavy meromyosin or subfragment-1. To compare myosin II heads of phosphorylated and dephosphorylated myosin II in this way will not be possible, however, because in making the analogues of muscle heavy meromyosin or myosin subfragment-1 (which thus far has not been possible) the tail portion of the myosin II molecule would be removed and that is the segment that contains the regulatory phosphorylation sites.

Both phosphorylated and dephosphorylated myosin II are monomers in 0.6 M KCl as measured by sedimentation-equilibrium. Both forms of the isoenzyme (20 μg/ml) remained soluble when dialyzed against 300 mM KCl, as measured by sedimentation in the Beckman Airfuge. About 85% of the phosphorylated myosin II remained non-sedimentable when the KCl concentration was lowered by dialysis to 25 mM. On the other hand, the solubility of dephosphorylated myosin dropped to 55% at 200 mM KCl and to 25% at 25 mM KCl. The sedimented myosin II was in bipolar filaments. These data suggest that self-association of myosin II is greater for the dephosphorylated, enzymatically active form of the enzyme than for the phosphorylated, enzymatically inactive form of the enzyme.

The effect of MgATP on the association of myosin II was assessed by changes in light scattering. Filaments of dephosphorylated and phosphorylated myosin II were first formed by dialysis against a buffer containing 20 mM KCl and 10 mM $MgCl_2$. As determined by light scattering measurements, the dephosphorylated myosin II was much more aggregated than the phosphorylated myosin II. Addition of MgATP to the phosphorylated myosin II caused a continuous gradual decline in light scattering to a very low final value at 3 mM MgATP. Addition of MgATP to the more highly aggregated dephosphorylated myosin II had a similar gradual effect until about 0.1 mM MgATP was reached after which there was a sharp decline in turbidity up to 3 mM MgATP. The final turbidity of the dephosphorylated myosin II in 3 mM MgATP was the same as the initial turbidity of the phosphorylated myosin II in the absence of MgATP.

The light scattering data were extended by negative staining electron microscopy. The dephosphorylated filaments changed from large bipolar aggregates with central bare zone thickness of about 20 nm in 3 mM MgATP. The

phosphorylated myosin initially formed bipolar filaments with a bare zone, 2 nm thick in the absence of MgATP and these became very small bipolar filaments with a bare zone less than 6 nm thick in 3 mM MgATP.

Thus, by all available criteria, dephosphorylated myosin II self-associates into larger filaments at higher concentrations of KCl and MgATP than does the phosphorylated form of myosin II.

4. Acanthamoeba Myosin II Heavy Chain Kinase: Problems of instability have made it very difficult to purify this enzyme much beyond the stage reported last year. Fortunately, the partially purified enzyme is sufficiently free of competing activities (phosphatases or kinases acting on contaminating proteins) that it can be used to phosphorylate myosin II for studies such as those just discussed. All of the proteins in the partially purified kinase preparation are smaller than myosin II, so myosin II can be recovered from the phosphorylation mixture simply by gel filtration on agarose columns.

5. Acanthamoeba Myosin II Phosphatase: Obviously, if regulation of the state of phosphorylation of the myosin II heavy chains is the physiologic control mechanism, there must exist a phosphatase to remove the phosphate residues as well as a kinase to put them on. To seek such a phosphatase, we prepared myosin II labeled with ^{32}P on the regulatory sites by incubating myosin II with [γ-^{32}P]ATP and partially purified myosin II heavy chain kinase (after first removing the endogenous phosphate moieties by incubating the myosin II with commercial potato acid phosphatase). The myosin II was freed of phosphatase, and then of kinase, by gel filtration. This ^{32}P-labeled myosin II could then be used to assay for heavy chain phosphatase activity by measuring the release of $^{32}P_i$. A very active phosphatase was obtained in the fraction eluted from a DE-52 column with 0.25 M KCl (just after the position of elution of myosins IA and IB). This fraction also contains actin but most of the actin can be removed by polymerizing it by the addition of MgATP. The phosphatase can then be precipitated and concentrated by 30% ethanol, dissolved in buffer, dialyzed to remove the ethanol and highly purified by eluting it from a chromatofocussing column with a gradient of KCl. The most purified material thus far obtained has mostly one band at a mobility very near to that of actin on an SDS-gel electrophoresis. It remains to be determined if this band represents the phosphatase or if the phosphatase is one of the several minor bands.

The partially purified phosphatase removes the phosphate groups approximately equally well from each of three phosphorylation sites on the heavy chains of myosin II. Accurate activity data have been difficult to obtain because the rate of dephosphorylation has not been directly proportional to enzyme concentration. The kinetics may be complicated by the presence of three different phosphate groups on each heavy chain. The enzyme is much less active toward phosphorylated light chains from smooth muscle myosin but phosphorylated light chains may nonetheless be a better substrate to obtain reliable kinetic data because of its simpler structure. The enzyme has no activity toward the non-protein substrate p-nitrophenylphosphatase.

6. Acanthamoeba Calmodulin: Although no evidence has ever been obtained that any of the Acanthamoeba myosin isoenzymes or the kinases that act on them are affected by Ca^{2+}/calmodulin, the possibilities remain that (a) an Acanthamoeba calmodulin would act differently from the mammalian brain calmodulin that has been tested, (b) a calmodulin-binding site on one of the Acanth-

amoeba enzymes has been lost making it calmodulin-independent; or (c) calmodulin might act through a different mechanism, for example to inactivate one of the heavy chain kinases or activate a myosin phosphatase. Therefore, it seemed of interest to attempt to purify and characterize an Acanthamoeba calmodulin, the more so because this ameba had been reported not to contain calmodulin.

In collaboration with Dr. Claude Klee, calmodulin has been purified essentially to homogeneity starting with about 200 g of amebas. The purified protein has an SDS-gel electrophoretic mobility significantly faster than brain calmodulin despite a very similar molecular weight by amino acid analysis. This is similar to the behavior of plant and protozoal calmodulins, in general. Its electrophoretic mobility is also faster in the presence of Ca^{2+} than, with EGTA as for other calmodulins. Its amino acid composition is very similar to those of Tetrahymena and spinach calmodulins and, similarly to all calmodulins, contains no tryptophan and 1 residue of trimethyllysine. The absence of tryptophan is also reflected in its ultraviolet absorption spectrum.

Acanthamoeba calmodulin activates brain phosphodiesterase with about the same K_D as for brain calmodulin but the maximal activation is only about 70% as great. The activity of the Acanthamoeba calmodulin on the ameba myosins and kinases has not yet been tested.

The purification of calmodulin involves Ca^{2+}-dependent affinity chromatography on a phenathiazine-Sepharose column followed by reverse phase HPLC. Calmodulin was separated only with great difficulty from another Ca^{2+}-dependent phenathiazine-binding protein of faster SDS-gel electrophoretic mobility. The amino acid composition of this protein indicates that it is not a degradation product of calmodulin and it has no activity in the phosphodiesterase assay.

Proposed Course of Research:

1. Myosin I Heavy Chain Kinase: We hope to complete the general characterization of this enzyme first by determining the optimal assay conditions and then determining the K_M and V_{max} values. The peptides of myosin IA and IB that are phosphorylated may be isolated and the phosphorylated amino acid identified. The kinase is known to autophosphorylate and the effect of this may be studied. Possibly antisera will be obtained with the intention of attempting localization studies. The availability of less than 1 mg of kinase makes structural studies very difficult.

2. Myosins IA and IB: The studies initiated this year on the actin-binding and actin-activation of phosphorylated and dephosphorylated myosin IA will be completed. Future studies may include one or more of the following: stopped flow kinetic studies to determine the intermediates in the ATPase cycle; structural studies to relate the ATPase site to the phosphorylation site; localization of the myosins in the cell by immunoelectron microscopy.

3. Myosin II: A major effort will be made to purify the 9000-dalton chymotryptic peptide (described in the annual report last year) that contains all three phosphorylation sites and to study its structure and the localization within it of the three phosphorylation sites. The relative positions of the ATPase and phosphorylation sites will be further studied (see last year's

annual report). The effect of phosphorylation on the association of myosin II with actin and on its self-association will be further studied. Cell localization of myosin II by immunoelectron microscopy may be attempted.

4. Myosin II Heavy Chain Kinase: Further studies are not presently planned.

5. Myosin II Heavy Chain Phosphatase: Efforts will be continued to purify this enzyme to homogeneity and then to study the nature of the phosphatase reaction. With the kinase and phosphatase it should be possible to inactivate and activate myosin II cyclically and thus model the presumed biologic regulation of the enzyme. The specificity of the phosphatase and its possible regulation will be studied.

6. Acanthamoeba Calmodulin: It is anticipated that the sequence of this calmodulin will be determined in collaboration with Dr. Tom Vanaman, Duke University. Its possible effects on the Acanthamoeba myosins, kinases and phosphatase will be studied.

Publications:

1. Cote, G.P., Collins, J.H., and Korn, E.D.: Identification of three phosphorylation sites on each heavy chain of Acanthamoeba myosin II. J. Biol. Chem. 256: 12811-12816, 1981.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00508-01 LCB

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Isolation and Characterization of Acanthamoeba Plasma Membrane.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Blair Bowers — Research Biologist — LCB NHLBI

Other: Barbara J. Clarke — Visiting Scientist — LCB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Section on Cellular Biochemistry and Ultrastructure

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

New procedures were developed for isolation of Acanthamoeba plasma membranes. Purity of the membrane fraction for which no unique enzymatic markers are known, was assessed by surface iodination. It was established that iodination labeled only the plasma membrane. About 26-fold purification of membranes was achieved. Purified plasma membranes will be examined for chemical differences with phagosome membranes.

PHS-6040
(Rev. 2-81)

Objectives: Our objectives were to develop a technique for the isolation of plasma membranes from Acanthamoeba and to develop a technique for specifically labelling plasma membrane proteins as a means of assessing the purity of the plasma membrane isolation. There is electron microscopic evidence that the plasma and phagosome membranes differ morphologically. This study is directed toward determining biochemical differences between the two membranes. Knowledge of the biochemistry of the two interchangeable membrane compartments will be valuable in gaining an understanding of the function of membrane components during endocytosis.

Methods Employed: Plasma membranes were isolated on density gradients using Percoll as the centrifugation medium. Iodination with IODO-GEN was performed to label membrane proteins. Polyacrylamide gel electrophoresis and autoradiography were used to study the protein composition of the plasma membrane and analyze the distribution of radioactivity among the membrane proteins. Standard biochemical procedures were used for protein and enzymatic determinations. Light and electron microscopy were used to monitor the isolation procedure and to determine the morphology of the final product.

Major Findings:

1. Iodination of Plasma Membrane: We have developed a reliable procedure for the radiolabelling of plasma membrane proteins of Acanthamoeba. We have determined that the radiolabel is restricted to plasma membrane proteins and is not internalized as there is no radioactivity in the actin-band of SDS-PAGE gels, as revealed by autoradiograms. (Actin is associated with the cytoplasmic side of the plasma membrane and stays with it during its isolation but is not considered to be an integral component of membranes as it is readily removed by chaotropic agents such as KI.)

In addition, the iodination procedure is very gentle to the cells as cell lysis was only 0.16%.

This procedure permits us not only to assess plasma membrane purity but will be a useful tool for further identification of membrane proteins.

2. Isolation of Plasma Membrane: We have obtained highly purified plasma membranes using a combination of differential and density gradient centrifugation on Percoll gradients. With this procedure, plasma membranes have been obtained that have up to a 38-fold (26-fold average) enrichment of radiolabel relative to the homogenate. There was little contamination of the membranes with lysosomes as determined by the presence of acid phosphatase activity (6% of specific activity of homogenate). An enrichment of alkaline phosphatase activity (7- to 10-fold over homogenate) was an indication of some contamination with contractile vacuoles. Electron microscopy also revealed the presence of contractile vacuoles in the plasma membrane preparation.

Significance to Biomedical Research: Several protective cells of the body such as macrophages, perform their function by the process of endocytosis. Knowledge of the events of this process will aid in treating or preventing diseases associated with a breakdown or alteration of normal endocytic events.

Proposed Course: We will supplement the characterization of membrane purity with enzyme assays for contaminants, and using previously developed methods for isolation of phagosome membranes, will isolate sufficient plasma and phagosome membranes for quantitative analysis of protein, phospholipid, sterol and lipophosphonoglycan content of the two types of membranes.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00509-01 LCB

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Lysosomes and Hydrolase Secretion in Acanthamoeba

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Section on Cellular Biochemistry and Ultrastructure

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Acanthamoeba has been shown to secrete lysosomal enzymes into the culture medium. This release of lysosomal enzymes was energy-dependent and was not due to cell lysis. The five hydrolases studied showed three patterns of secretion: (a) secretion of acid phosphatase and β-glucosidase was stimulated by phagocytosis of nutrient particles, but unaffected by phagocytosis of indigestible particles; (b) secretion of N-Acetyl-β-hexosaminidase and esterase was unaffected by phagocytosis of nutrient particles and was inhibited by phagocytosis of latex beads; (c) secretion of α-galactosidase was stimulated by phagocytosis of both nutrient and indigestible particles. These results are interpreted to be evidence for subpopulations of lysosomes that fuse variably with phagosomes and/or plasma membranes.

PHS-6040
(Rev. 2-81)

Objectives: Using Acanthamoeba as a model system, our long-range objectives are to understand the mechanisms involved in the exchange between internal membrane systems and the plasma membrane. The kinetics and physiology of lysosomal enzyme secretion is one window on membrane exchange since the hydrolases clearly enter the cell's internal digestive compartment and are also released into the external medium. Thus, it is likely that they serve as a "marker" for membrane movement. This year the major thrust has been toward characterizing the release of lysosomal enzymes under a variety of experimental conditions.

Major Findings:

1. Measurement of Rates of Digestion: In order to obtain preliminary evidence on rates of lysosomal fusion with phagosomes, amebas were fed yeast that had been grown on ^{35}S-methionine. Amebas were allowed to ingest 7, 13, and 30 yeast/cell, washed and reincubated to follow the digestion of yeast. Total radioactivity was obtained as a function of time in three compartments: (1) activity remaining associated with yeast; (2) activity released into the medium (was not TCA precipitable); and (3) activity incorporated into ameba protein. The curve of incorporation of radioactivity into ameba protein was sigmoidal, i.e. there was a lag as phagosomes acquired hydrolases, then a maximal rate of incorporation from one to two hours after the wash, followed by a decreased rate as the available substrate disappeared. The rate of incorporation into ameba protein was proportional to the load between 7 and 13 yeast/cell, but the rate with 30 yeast/cell was similar to that at a load of 13/cell. This result suggests that the rate limit may be imposed by a fixed capacity for digestion (presumably determined by available hydrolase). A number of attempts to perturb the digestion of yeast by agents known to interfere with lysosomal function in mammalian cells had no effect on Acanthamoeba, even though the compounds were applied at relatively high concentration. We attribute the lack of effect to the relative impermeability of Acanthamoeba plasma membrane and to the inefficiency of pinocytic uptake.

2. Identification of Lysosomal Enzymes and Conditions for Assay. Homogenates of Acanthamoeba were surveyed for the following enzyme activities: acid phosphatase, N-acetyl-β-hexosaminidase, β-glucosidase, β-glucuronidase, α-galactosidase, acid ribonuclease, esterase, aryl sulfatase 1 and 2, and tri-meta-phosphatase. No aryl sulfatase or tri-meta-phosphatase activity was detected. The pH optimum, the appropriate buffer and assay condition was determined for each of the remaining seven enzymes. Fluorogenic substrates were found to give adequate sensitivity for enzymes of low activity. Five of the seven enzymes were used in the study described below.

3. Secretion of Lysosomal Enzymes. Release of hydrolases into the medium occurs at a low but detectable rate. The rate of secretion of five enzymes was characterized under three conditions: pinocytosis, phagocytosis of yeast and phagocytosis of beads. In all cases the rate of secretion was linear with time, but secretion rates were not identical. In the presence of 10^{-3} M sodium azide there was no secretion of enzymes. Tests for cell lysis showed generally less than 1% cell lysis occurred during experiments. In bead experiments lysis was somewhat higher but was a maximum of 4%. Under conditions where the rate of pinocytosis was about 50% of that in culture medium, none of the hydrolase

activities were detected. These controls indicate that the secretion is dependent on an active process related to endocytosis.

The secretion patterns of the five enzymes under the three experimental conditions showed interesting differences. In pinocytosing cells acid phosphatase (AP), β glucosidase (β-glu) and α galactosidase (α-gal) were secreted at a rate of 3% of the total activity per hour. Two of the five enzymes, N-acetyl-hexosaminidase (NAH) and esterase (EST) were secreted at a rate of 14% per hour.

In phagocytosing cells the secretion rate changed after saturation with particles. After saturation of cells with yeast all enzymes were secreted at a rate of about 11% of the total activity per hour. On the other hand, after saturation of cells with beads secretion of AP and β-glu remained at about 3%, NAG and EST dropped from 14% to 3% and α-gal increased to 15%. From these results, it appears that there are at least three distinct patterns of hydrolase release: one pattern is demonstrated by AP and β-glu, which are stimulated by the ingestion of yeast but unaffected by the ingestion of latex beads; a second pattern demonstrated by NAG and EST activities, in which hydrolase secretion is inhibited by the ingestion of both yeast and beads, with beads being the more effective inhibitor; the third pattern, exemplified by α-gal, which is stimulated by ingestion of both yeast and latex beads. These observations are tentatively interpreted to indicate there may be subpopulations of lysosomes in Acanthamoeba which vary in the frequency with which they fuse with the plasma membrane and/or phagosomes.

Significance to Biomedical Research: Lysosomes have long been known to be heterogeneous organelles. It is unknown, however, if the subpopulations participate in separate catabolic processes or merely represent different stages of organelle maturation. Moreover, lysosomal dysfunction has been implicated in a large number of mammalian diseases including genetic disorders as well as parasitic infections. Understanding normal lysosomal functioning is an obvious prerequisite for treatment of these diseases.

Amebas are not unique in their ability to secrete lysosomal hydrolases. Among other cells, mammalian fibroblasts and macrophages also secrete these activities. While some phagocytic stimuli have been shown to affect the rate at which these hydrolases are released in certain systems, there are conflicting reports in the literature. The present work demonstrates that hydrolase release is a function not only of the type of phagocytic stimuli, but that the pattern of secretion of individual or groups of hydrolases may vary.

Proposed Course of the Project: Having characterized secretory patterns for lysosomal enzymes in Acanthamoeba, the next effort will be to determine the source and route of secretion. We will attempt to identify and characterize lysosomal subpopulations by density gradient separations. Electron microscopic cytochemistry will be used to localize two enzymes of different secretory behavior in order to determine if different pathways are evident.

Publications:

None

PROJECT NUMBER (Do NOT use this space)	HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	Z01 HL 00510-01 LCB

PERIOD COVERED
October 1, 1982 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Nucleotide-Induced Conformations of the Acto·S-1 Complex

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	Lois E. Greene	Research Chemist	LCB	NHLBI
	Evan Eisenberg	Section Head, Cellular Physiology	LCB	NHLBI

COOPERATING UNITS (if any)

None

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SECTION
Cellular Physiology

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.08	3	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The effect of the troponin-tropomyosin complex on the binding of S-1 to actin has provided evidence for two different conformations of the acto·S-1, depending on the nucleotide bound to S-1. In the presence of ADP or the nucleotide analog, AMP-PNP, S-1 binds to regulated actin tightly and with positive cooperativity. However, under the same conditions, the binding of S-1 to regulated actin in the presence of ATP is much weaker and appears non-cooperative. By examining the binding of S-1·ATP and S-1·AMP-PNP to regulated actin under conditions where they bind with equal affinity to actin, we found that S-1·AMP-PNP binds cooperatively to regulated actin, whereas S-1·ATP does not. These results suggest that the structure of the acto·S-1 complex is different with ATP and AMP-PNP. We have further shown that a stable analog of the S-1·ATP state can be formed by cross-linking two cysteine groups of the S-1 molecule with N,N'-p-phenylenedimaleimide. As does S-1·ATP, the cross-linked S-1 binds very weakly to actin and does not exhibit significant cooperativity in binding to regulated actin.

PHS-6040
(Rev. 2-81)

Objectives: Most models of muscle contraction suggest that, during the ATPase cycle, there is a change in conformation of the actin-bound cross-bridge associated with the step where P_i is released from the actomyosin. Since the nucleotide species bound to S-1 is thought to affect the conformation of the acto·S-1 complex, we examined whether the binding of different S-1 nucleotide complexes to regulated actin is evidence for different conformational states. This was based on our previous studies in which we found that troponin-tropomyosin markedly affects the binding of S-1 to actin in the presence of ADP or AMP-PNP, but does not significantly affect the binding in the presence of ATP. In addition, we determined whether S-1 which had been modified using the bifunctional cross-linking reagent, N,N'-p-phenylenedimaleimide has a conformation resembling that of S-1·ATP or S-1·ADP.

Methods Employed and Major Findings: The binding of S-1 to regulated actin in the presence of ATP was determined either by measuring the instantaneous increase in turbidity on a stopped-flow spectrophotometer or by measuring the unbound S-1 in the supernatant by an ATPase assay after separating it from the actin-bound S-1 by centrifugation. The binding of S-1·AMP-PNP to regulated actin was measured by using S-1 which had its SH_1 group blocked with [^{14}C]-iodoacetamide. In this case, the S-1 not bound to actin after centrifugation was determined by measuring the radioactivity of the supernatant. In all cases, the binding was measured as a function of the free S-1 concentration. The binding studies were conducted at 18 mM and 180 mM ionic strength in the presence of ATP and AMP-PNP, respectively, conditions in which the S-1 bound to actin with equal affinity with both nucleotides ($K = 1.4 \times 10^4\ M^{-1}$). In the presence of AMP-PNP, the binding of S-1 to regulated actin was weak at low concentrations of free S-1 and increased in a positive cooperative manner as the concentration of free S-1 was increased and the S-1 sites on actin became occupied. In contrast, the binding of S-1 to regulated actin in the presence of ATP had a constant strength of binding over a very large range of free S-1 concentration, showing no evidence of cooperativity. These data support a model in which S-1 can attach to actin in at least two ways. In the presence of ATP, the binding occurs in such a way that the affinity is insensitive to changes caused by the increased saturation of actin with S-1. On the other hand, in the presence of AMP-PNP (or ADP), binding occurs in a different way, making the binding of S-1 to actin sensitive to changes in the conformation of the troponin-tropomyosin-actin complex. These two binding states may correspond to the two attached states of the actomyosin which have been proposed to explain force generation in muscle.

In the hope of finding a stable analog of S-1·ATP, we have also examined the binding of S-1 to actin after cross-linking the SH_1 and SH_2 cysteines on S-1 with pPDM. This was done by modifying the S-1 with [^{14}C]-pPDM, which we had synthesized, and then measuring the binding to actin both in the absence and presence of the troponin-tropomyosin complex. We found that in all respects, pPDM·S-1 closely resembles S-1·ATP and is very different from S-1·ADP, i.e. its affinity to actin is extremely weak and there is only a slight effect of troponin-tropomyosin on the binding of pPDM·S-1 to actin. Therefore, the conformation of pPDM·S-1 resembles the conformation induced by the binding of ATP on S-1.

Significance to Biomedical Research: One of the key questions in understanding the mechanism of muscle contraction is the relationship between protein structure and energy transfer. We have obtained insights into this question, which is of great importance in all energy transducing systems. This work is potentially significant in muscle disorders, including those of skeletal, cardiac, and smooth muscle.

Proposed Course of Research: The plan, in this project, is to examine whether there are more than two conformations of the acto·S-1 complex, perhaps an intermediate state between the S-1·ATP and S-1·ADP. This will be done by modifying S-1 with cross-linkers of different lengths. In addition, since nucleotides are able to bind to the active site after cross-linking, we will determine the effect of different nucleotides bound to the cross-linked S-1. In all cases, the ability of the cross-linked S-1's to bind to actin both in the presence and absence of troponin-tropomyosin will be examined. Furthermore, the fluorescence change induced by the binding of these cross-linked S-1's to regulated actin will be measured (see Project No. Z01 HL 00413-06), to determine whether the tropomyosin can exist in more than two positions on the actin filament.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 00511-01 LCB

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Identification of Free Radicals in Irradiated Aqueous Solutions of Biological Macromulecules

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	A. Carmichael	Visiting Fellow	LPP	NCI
	P. Riesz	Head, Radiation Biology Section	LPP	NCI

COOPERATING UNITS (if any)

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SECTION

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INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.25	0.25	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The role of free radical chemistry in many biological processes is becoming increasingly recognized. There are at present, however, no satisfactory methods for studying the distribution of radical content on biological macromolecules. The present effort may afford a means for accomplishing this objective. Thus, an aqueous solution of the macromolecular sample (presently studied are lysozyme and ribonuclease) is γ-irradiated in the presence of a spin-trapping reagent to stabilize the transient free radical content that results from irradiation. The sample is degraded proteolytically, under conditions that ensure maintenance of the radical content. The resulting amino acids (and possibly small peptides) may then be separated, identified, and radical content of each component studied by electron spin resonance (e.s.r.). Initial efforts in producing digests, in which the radical content has been maintained, have proven successful. Further efforts will be directed toward separation and e.s.r. study of the radical-containing components.

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PHS-6040
(Rev. 2-81)

Objective: To study the free radical distributions on biological macromolecules in aqueous solution after irradiation. The radical content is to be stabilized by a "spin trapping" reagent. Means will then be sought to degrade the labelled sample under conditions that will maintain the radical content. The resulting fragments will be separated and studied by electron spin resonance (e.s.r.).

Methods:

1. Irradiation of aqueous protein solutions is carried out in a Co^{60} source.

2. Free radical content resulting from irradiation is studied with a Varian E3 electron spin resonance spectrometer.

3. A "spin trapping" reagent is included during irradiation. In this method, the short-lived radicals initially produced by irradiation are stabilized by reaction with 2-methyl-2-nitroso-propane, and the product is then readily studied by e.s.r. It can then be subjected to certain degradative procedures with minimal loss of radical content.

4. An original procedure is used for the enzymatic hydrolysis of the irradiated protein, involving digestion successively with pepsin, pronase, prolidase, and leucine aminopeptidase.

5. Amino acid analysis (Beckman Amino Acid Analyzer, Model 120) is employed to determine the extent of enzymatic hydrolysis by the yield of resulting amino acids.

Findings: A means for the irradiation of aqueous lysozyme and ribonuclease has been developed, sufficient to produce a free radical content that can be studied by e.s.r. after "spin-trapping." A proteolytic method has been developed, employing limited pepsin digestion as an initial step. Despite the known deleterious effects of low pH on the spin-trapped radical content, conditions of time and temperature have been worked out so as to minimize loss of radical content. There follow digestions with pronase, prolidase, and leucine aminipeptidase, designed to maximize the extent of hydrolysis while minimizing the loss of radical content. An initial separatory procedure with a gel filtration technique indicates that approximately 40% of the resulting samples, from digestion of lysozyme and ribonuclease, appears as a peak containing amino acids, and possibly low molecular weight peptides.

Significance to Biomedical Research: It is becoming increasingly recognized that free radical chemistry plays a significant role in biology. Aside from the obvious significance of radiation damage, there are naturally occurring processes in phagocytosis inflammation, hemolysis, lipid peroxidation, and mitochondrial respiration involving free radical mechanisms through the generation of hydroxyl radicals and superoxide anions. A recent review is given by W. Bors et al., Photochem. and Photobiol. 28, 629 (1978). Thus, it is becoming increasingly desirable to develop methods for detection and location of radical content. There are, at the present time, no satisfactory methods for studying radical content on biological macromolecules. The present effort, if successful, will afford such a method.

Proposed Course of Project: Further methods will be explored for the separation of the amino acids and peptides resulting from proteolytic digestion of irradiated and "spin-trapped" proteins, followed by e.s.r. examination, to determine the radical distribution within the original molecule. If this technique is successful, the investigation will be expanded to include other proteins and nucleic acids.

Publications:

None.

ANNUAL REPORT OF THE
LABORATORY OF CELLULAR METABOLISM
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

October 1, 1981 to September 30, 1982

Research in the Laboratory of Cellular Metabolism continues to be largely concentrated on the enzymes responsible for the synthesis and degradation of cAMP and cGMP through which many hormones, drugs, and other agents influence cellular function. Some change in emphasis has occurred during the past year with expansion of work on the ADP-ribosyltransferases of animal cells and removal of the Section on Cellular Pharmacology to the Laboratory of Chemical Pharmacology.

1. Cyclic Nucleotide Phosphodiesterases

Calmodulin-activated phosphodiesterase has now been purified from ovine as well as bovine brain. Both enzymes are equally sensitive to calmodulin and are activated to similar extents. The two enzymes are, however, clearly different in primary structure as indicated by amino acid composition and proteolytic fragmentation. Both enzymes are proteins of ~ 59,000 daltons (monomer). The bovine enzyme is sequentially cleaved by chymotrypsin to yield 57,000 and 45,000 dalton products, whereas the ovine enzyme is degraded to 55,000, 53,000, and 38,000 peptides. The final products of chymotryptic cleavage are resistant to further action of the enzyme and exhibit catalytic activity equal to that of the native enzyme assayed in the presence of calmodulin. Treatment with chymotrypsin in the presence of calmodulin also produced enzyme that was fully active in its absence, but the final stable peptides were 47,000 (bovine) and 43,000 daltons (ovine), indicating that when the phosphodiesterase interacts with calmodulin a new site, protected in the absence of calmodulin, becomes susceptible to chymotryptic cleavage.

Last year, we initiated use of dansyl-calmodulin, a fluorescent derivative, to characterize the interaction of calmodulin with its binding proteins. Preparations of dansyl-calmodulin are apparently identical to native calmodulin in ability to activate phosphodiesterase, and > 85% of the dansyl fluorescence is found in one CNBr peptide, consistent with selective modification of one calmodulin domain. Ca^{2+}-dependent changes in the fluorescence of dansyl-calmodulin were markedly influenced by Mg^{2+} and NaCl. Increasing ionic strength reduced affinity for Ca^{2+}. In the absence of Mg^{2+}, increasing Ca^{2+} first increased fluorescence intensity in a manner suggestive of positively cooperative Ca^{2+} binding followed by a decrease in fluorescence. With > 5 mM Mg^{2+}, the decrease did not occur, consistent with the view that Mg^{2+} competes for lower affinity Ca^{2+}-binding sites. We conclude that maximal fluorescence accompanies occupation of the first two sites; the decrease corresponds to filling the third and fourth sites. Collaborative studies of circular dichroism reflected conformational changes concomitant with the decrease in fluorescence. It appears that at least three different conformers of calmodulin exist in solution depending on the number of Ca^{2+} sites occupied. With addition of troponin I, calcineurin, myosin light chain kinase, or the phosphodiesterase (in the presence of Ca^{2+}), fluorescence intensity was increased proportional to the amount of binding protein added and was maximal at 1 mol per mol calmodulin. The Ca^{2+} dependence of the increase in fluorescence was not altered by any of the binding proteins. Polarization of fluorescence demonstrated, for the first time, that complex formation with a binding protein accompanies the "cooperative" binding of Ca^{2+} to the two high

affinity sites of dansyl-calmodulin. Whether complex formation in itself, with two calmodulin Ca^{2+}-binding sites occupied, is sufficient to alter function of a binding protein remains to be determined.

A new affinity matrix for purification of the phosphodiesterase (and other calmodulin-binding proteins) was developed. As calmodulin contains no sulfhydryl groups, the pyridyldithiopropriony1 derivative was prepared and from it disulfide-linked calmodulin-Sepharose. The affinity gel was used to bind phosphodiesterase (and other calmodulin-binding proteins); the bound proteins and calmodulin were quantitatively released with dithiothreitol and separated by gel filtration. Binding capacity of the affinity gel for phosphodiesterase was ~ five times that of conventionally prepared calmodulin-Sepharose and recovery of activity was reproducibly high. This general strategy should prove useful for other types of affinity chromatography.

Several years ago, we described some characteristics of a so-called cGMP-stimulated phosphodiesterase. An enzyme of this type has now been purified to homogeneity (~ 13,000-fold) from calf liver supernatant. The native enzyme appears to be a nonspherical dimer of very similar, if not identical, ~ 100,000 dalton peptides. It hydrolyzes cAMP and cGMP with kinetics suggestive of positive cooperativity.

In addition to studies of the molecular mechanism for regulation of catalytic activity, we are investigating conditions that influence its activity in cultured dog kidney (MDCK) cells. Normal cells contain calmodulin-stimulated and cAMP phosphodiesterases but virtually none of the cGMP-stimulated enzyme, which is present, however, after cells are transformed with Harvey sarcoma virus. (Transformation also alters amounts of the other phosphodiesterases.) Others have shown that butyrate, methylxanthine, or PGE_1 can restore some differentiated characteristics (e.g., glucagon receptors) missing in transformed cells. None of these restored the "normal" pattern of phosphodiesterases, and butyrate specifically increased the amount of cGMP-stimulated activity. Elucidating the mechanism of this effect should provide further understanding of how amounts of individual phosphodiesterases are regulated in cells and perhaps provide clues to the roles played by the several members of this family of enzymes that is responsible for terminating the signals (cyclic nucleotides) that mediate the effects of so many hormones and neurotransmitters.

We earlier reported on a particulate phosphodiesterase in rat adipocytes that is activated by insulin and obtained evidence that its activity could be influenced by glucocorticoids, e.g., dexamethasone, although this could not be directly demonstrated in vitro because of the limited viability of the isolated cells. To investigate the effects of dexamethasone (and other slow-acting agonists), we have now initiated experiments with cultured 3T3-L1 fibroblasts that differentiate into cells with morphological and enzymatic characteristics of mature adipocytes. With differentiation, a particulate cAMP phosphodiesterase becomes responsive to insulin. In 3T3-L1 adipocytes, physiological concentrations of insulin caused a rapid and specific increase in this activity which was also increased rapidly and transiently by hormones (epinephrine, ACTH, TSH) that cause lipolysis (as we observed earlier in rat fat cells). Incubation of 3T3-L1 adipocytes with < 1 μM dexamethasone for 2-3 days reduced or abolished responsiveness to insulin and epinephrine. Current studies are focused on the means through which glucocorticoids modulate these acute effects of insulin and hormones that activate adenylate cyclase.

2. Guanylate Cyclase

Using our highly purified rat liver guanylate cyclase, which, unlike crude enzyme, cannot be activated by NO, we are attempting to define the mechanism of activation by NO and other compounds. Guanylate cyclase activity requires Mg^{2+} or Mn^{2+}. Basal activity of the purified enzyme was higher with Mn^{2+} but dependence on GTP concentration was complex, whereas with Mg^{2+} double reciprocal plots were linear. In assays with Mg^{2+}, activity could be increased severalfold by activator (as we have reported) or protoporphyrin IX; further addition of NO had no effect. Deoxyhemoglobin (Hb) slightly increased activity, but in its presence NO activation was > 50-fold. The enzyme was rapidly inactivated at 0°C and this was prevented by activator or Hb but not by NOHb or protoporphyrin. Further studies with a number of activators and inhibitors suggest that, although oxidation is involved in activation by NOHb, protoporphyrin, or activator, the mechanisms are different. In addition, certain compounds may enhance guanylate cyclase activity and stability because of their hydrophobic properties.

3. Adenylate Cyclase

Activation of adenylate cyclase by choleragen in intact cells requires initial binding of the toxin B subunits to ganglioside G_{M1} in the membrane. Subsequently, the A_1 peptide catalyzes the ADP-ribosylation of a guanyl nucleotide-binding protein (G/F) resulting in enhanced cyclase activity. To understand the mechanism by which the A_1 peptide gains access to G/F and explain the persistence of the toxin effect on cells, we monitored the fate of ^{125}I-choleragen bound to cultured fibroblasts and neuroblastoma cells. The A_1 peptide was apparently degraded more rapidly ($t_{1/2}$ ~ 1 day) than the B subunits ($t_{1/2}$ > 5 days) by fibroblasts. After exposure to toxin, cells recovered ability to bind ^{125}I-choleragen with a half-time of ~ 7 days. From these and other data, it appears that choleragen is degraded relatively slowly and remains bound to the surface of the cells for prolonged periods, which may explain the persistence of adenylate cyclase activation by the toxin. Studies of receptor–ligand interactions usually employ a "binding" step followed by extensive washing to remove free ligand. This, by definition, removes reversibly bound ligand. Equations usually used to analyze data, however, require equilibrium between free and bound ligand and are not applicable with such assays. Assuming that ligand-receptor interaction involves a reversible step followed by an "irreversible" event, the K_a for the reversible reaction may be obtained from the rate of the "irreversible" step. Interaction of choleragen with fibroblasts appears to be an example of "irreversible" binding. The two-step model was applied to determine the K_a for the reversible step in ^{125}I-choleragen binding to human fibroblasts at 0°C and 37°C. As predicted by the model, addition of unlabeled toxin enhanced release at 37°C; the rate remained low at 0°C. The two-step model for ligand binding should be useful also for analysis of other ligand-receptor interactions.

E. coli heat-labile enterotoxin (LT) is structurally, functionally, and immunologically similar to choleragen. In collaborative studies, we showed that LT and choleragen have the same specificities for interaction with ganglioside G_{M1} and its oligosaccharide. In addition, we found that after trypsinization the ADP-ribosyltransferase activity of LT (which we had previously demonstrated) is equivalent to that of choleragen. Activity of both toxins is enhanced by dithiothreitol, but choleragen is not activated by trypsin. The choleragen A subunit is synthesized as a single chain, but in most toxin preparations it exists as two disulfide-linked peptides, i.e., it has already been proteolytically cleaved.

The A subunit of LT is a single peptide chain; it appears that only after proteolytic cleavage can reduction of the disulfide bond release a peptide with full catalytic activity. Differences in the susceptibility of the two toxins to proteolysis could contribute to differences in their toxicity.

During the past year, most of our work on adenylate cyclase itself has been focused on individual components of the system, the catalytic unit C and the guanyl nucleotide-binding protein G/F, that is involved in activation by choleragen and hormones. Whether the reported activation of brain adenylate cyclase by calmodulin requires the presence of G/F has been in question. C, solubilized using CHAPS, a zwitterionic detergent, and separated from G/F, was activated by calmodulin, and activation was enhanced by phospholipids. Both calmodulin and phospholipids also slowed thermal inactivation of C. As the solubilized C was free of detectable functional G/F, it appears that calmodulin can activate C independent of G/F. In preliminary experiments, calmodulin-dependent and independent fractions of C are being separated. It is generally accepted that C activity is enhanced by G/F when the regulatory component has bound GTP; hydrolysis of GTP to GDP makes an inactive complex, and hormones activate cyclase by accelerating GDP release, thereby promoting GTP binding. Last year, we suggested that choleragen might also enhance release of GDP from G/F in turkey erthrocyte membranes. We have now shown that, following ^{32}P-ADP-ribosylation of G/F with choleragen (monitored as radiolabeling of the 42,000 dalton G/F peptide), GDP release from a protein complex containing cyclase activity and the ^{32}P-peptide is increased. The rate of release is increased more by epinephrine than by choleragen, consistent with differences in the effects of these two agents on adenylate cyclase activity. Thus, both may activate cyclase, at least in part, by decreasing the affinity of G/F for GDP. We had reported that the rapid inactivation of choleragen-activated brain adenylate cyclase at 30°C was prevented by GTP. That inactivation is not due to the removal of ADP-ribose from G/F has now been shown with fibroblast cyclase and with G/F in human erythrocytes that lack C.

In addition to receptors for agonists that activate adenylate cyclase, many cells have receptors for inhibitory ligands. These inhibitory receptors may be coupled to the cyclase through a guanyl nucleotide-binding protein (GI) distinct from G/F. To elucidate the mechanism of cyclase inhibition by acetylcholine, we have begun to isolate and characterize the muscarinic receptor-GI complex from cardiac membranes. Conditions for achieving stable coupling of receptor and GI and for solubilization of the coupled complex have been optimized.

4. ADP-ribosyltransferases

After demonstrating the ADP-ribosyltransferase activity of choleragen, we looked for analogous enzyme activity in animal cells. A transferase that catalyzes the same model reactions was found in turkey erythrocyte cytosol and purified to homogeneity. Similar enzymes have now been identified in several tissues. The turkey enzyme is activated by salts that promote dissociation of relatively inactive oligomers to protomers with high activity, and recent data show that histone activation also results from dissociation. The transferase is rapidly inactivated by diethyl pyrocarbonate (DEP) at 0°C. Our data are consistent with the conclusion that DEP modifies a single class of histidyl residues. Addition of 300 mM NaCl, which increased activity > 400%, increased k_{obs} for inactivation only ~ 70%, suggesting that structural requirements for accessibility of sites through which DEP inactivates are different from those for substrate accessibility and catalytic function. Since we had earlier found that choleragen-catalyzed

ADP-ribosylation of several proteins was enhanced by nucleoside triphosphates, we assessed their effects on transferase activity with a series of protein substrates. GTP was most effective nucleotide with choleragen but ATP was more effective for the transferase. With the same protein substrate, different effects of nucleotides were observed with the two enzymes, consistent with previously noted differences in their substrate specificities. During purification of the "histone-dependent" transferase, another activity unaffected by salt or histone was detected and has now been purified to homogeneity for further characterization.

Many ADP-ribosyltransferases also exhibit NAD glycohydrolase (NADase) activity, i.e., in the absence of other substrate they use water as an ADP-ribose acceptor. In fact, at least some NADases may be transferases for which specific acceptors have not been identified. We have partially purified from rat liver cytosol an NADase that is dependent on histones for activity. Although a number of NADases have been described, effects of histone or similar effectors have not been reported. This NADase exhibits no detectable ADP-ribosyltransferase activity and, in contrast to the transferases we have studied, catalyzes a pyridine base exchange. Its function like that of other NADases remains to be established.

5. Regulation of cAMP Metabolism in Intact Cells

Human fibroblasts respond to bradykinin (BK) with increases in production of PGE_2, PGI_2, and cAMP. PGE_2 and PGI_2 activate fibroblast adenylate cyclase, and all evidence indicates that the effect of BK on cAMP is secondary to its enhancement of prostaglandin synthesis. Growth conditions markedly influence the magnitude of the BK effects; our data suggest that this is due to alterations in the ability of BK to increase prostaglandin production. In other experiments, we found that elevation of intracellular cAMP decreased the prostaglandin response to BK. Thus, this effect of BK may be modulated via feedback inhibition by cAMP. Completed studies of bradykinin binding and metabolism by intact cells permit us to begin to define factors that modify BK receptor number and function. Investigations of BK effects on phospholipase activity and phospholipid metabolism have been initiated.

6. Regulation of Lipid Metabolism in Mammalian Cells

Last year, collaborative studies on the regulation of cholesterol synthesis and the activity of the rate-limiting enzyme hydroxymethylglutaryl Coenzyme A reductase demonstrated that, in addition to slow effects on reductase activity in cultured cells, mevalonate and low density lipoprotein produced rapid inactivation of the enzyme by promoting its phosphorylation. Several other known inhibitors of cholesterol synthesis have now been tested. Glycocholic acid, 25-hydroxycholesterol and 7-ketocholesterol each caused rapid, reversible inhibition; total activity was decreased only with longer exposure.

Cultured fibroblasts from three patients with adrenoleukodystrophy contained larger amounts of very long chain fatty acids (VLCFA) than did normal fibroblasts as reported by others. To determine whether this is due to a defect in catabolism, rates of oxidation of VLCFA and palmitate were determined. Cells from five patients oxidized all fatty acids more slowly than did normal cells, but the difference was greater for VLCFA. It has been suggested that oxidation of VLCFA is extramitochondrial and thus subject to a defect that does not interfere with metabolism of common long chain fatty acids. Results of our experiments suggest, however, that VLCFA are metabolized in the mitochondrion and that the patients' cells are normally responsive to carnitine.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00603-08 CM

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Regulation of Cyclic Nucleotide Phosphodiesterase Activities in Cultured Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Vincent C. Manganiello	Head, Section on Biochemical Physiology	CM	NHLBI
	Toshihiko Yamamoto	Visiting Fellow	CM	NHLBI
	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI

COOPERATING UNITS (if any)

Dr. Michael Lin, Laboratory of Nutrition and Cellular Endocrinology, NIAMDDK, NIH.

LAB/BRANCH
Cellular Metabolism

SECTION
Biochemical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	0.6	0.6

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Supernatant (100,000 x g, 45 min) fractions from cultures of normal dog kidney (MDCK-N) and cells transformed by Harvey murine sarcoma virus (MDCK-T) contained both cyclic AMP (cAMP) and cyclic GMP (cGMP) phosphodiesterase activities. MDCK-N cells exhibited much greater cAMP and cGMP phosphodiesterase activities (assayed at 0.5 μM substrate) than MDCK-T cells. MDCK-T cells contained some cGMP-stimulated cAMP phosphodiesterase activity which was not detected in supernatants from MDCK-N cells. Incubation of MDCK-T cells with sodium butyrate markedly increased cGMP-stimulated cAMP hydrolytic activities. This effect was not observed in MDCK-N cells. Maximal effects were observed after incubation of MDCK-T cells for 24 h with 1-2 mM sodium butyrate, concentrations known to induce differentiated cell functions in these cells and other cell lines. MDCK-N cells contain calcium- and calmodulin-stimulated phosphodiesterase activity. MDCK-T cells contain much less calmodulin-sensitive activity; incubation with butyrate did not restore this activity in MDCK-T cells. Thus, MDCK cells may provide a useful system to study the regulation of various types of cyclic nucleotide phosphodiesterase activities.

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PHS-6040
(Rev. 2-81)

Project Description:

Objectives: To study mechanisms whereby cyclic nucleotide phosphodiesterase activity is regulated in eukaryotic cells. Specifically to study regulation of a cyclic GMP-stimulated cyclic nucleotide PDE in normal MDCK cells and a cloned line of transformed MDCK cells in which certain differentiated functions can be restored during incubation with sodium butyrate.

Methods Employed: MDCK cells were grown in culture to confluence. Cells were incubated for various periods without or with butyrate, harvested, and homogenized in a hypotonic buffer. Homogenates were centrifuged (100,000 x g for 45 min) and supernatant and particulate fractions analyzed for protein content and assayed for phosphodiesterase activity by a procedure developed earlier in this laboratory.

Major Findings: $MDCK_N$ cells contain glucagon receptors; incubation of these cells with glucagon is accompanied by a marked increase in cell cAMP content. In a cloned line of cells transformed by Harvey murine sarcoma virus ($MDCK_T$), the number of receptors for and the response to glucagon is markedly reduced. Incubation of $MDCK_T$ cells with sodium butyrate increases receptor number and restores responsiveness to glucagon.

Supernatant fractions (100,000 x g) fractions from $MDCK_N$ and $MDCK_T$ cells contain cAMP and cGMP phosphodiesterase activities. Activities in $MDCK_N$ cells are much higher than in $MDCK_T$ cells. Chroamtography on columns of AcA 34 and DEAE BioGel indicate that different types of phosphodiesterases are present in $MDCK_N$ and $MDCK_T$ cells. $MDCK_N$ cells contain a calcium and calmodulin-sensitive enzyme and cAMP phosphodiesterasse, $MDCK_T$ cells contain much less calmodulin-sensitive and cAMP phosphodiesterase activities. In addition, these cells contain a cGMP-stimulated cAMP phosphodiesterase.

Incubation of $MDCK_T$ cells with sodium butyrate (0.5-2.0 mM) for 8-36 h results in an increase in soluble and particualte cGMP phosphodiesterase activity and a decrease in cAMP PDE activity. Incubation of $MDCK_N$ cells with butyrate did not increase cGMP PDE activity. Kinetic analysis of fractions separated by chromatography in AcA 34 indicate that the cGMP-stimulated enzyme is specifically increased by incubation with butyrate. Other agents capable of restoring differentiated function (i.e., restoration of the glucagon receptor) in $MDCK_T$ cells such as PGE_1 and IBMX did not alter the activity of the cGMP-stimulated enzyme.

Significance to Biomedical Research: Cyclic nucleotides are important mediators of the effects of a number of hormones and pharmacological agents. By hydrolyzing cyclic nucleotides, phosphodiesterases presumably play an important role in termianting the signals produced by these effectors. Understanding mechanisms involved in regulation of the expression of these enzymes should be important in understanding how fundamental metabolic processes are controlled by hormones and pharmacological agents.

Proposed Course: Continued study of the regulation of the expression of these enzymes in cultured cells.

Publications: Vaughan, M., Danello, M.A., Manganiello, V.C., and Strewler, G.J.: Regulation of cyclic nucleotide phosphodiesterase activity. In Dumont, J.E., Greengard, P., and Robison, G.A. (Eds.): Advances in Cyclic Nucleotide Research. Raven Press, New York, 1981, Vol. 14, pp. 263-271.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00606-11 CM

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Regulation of cAMP Content and Prostaglandin Production of Cultured Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Carole L. Jelsema	Senior Staff Fellow	CM	NHLBI
	Vincent C. Manganiello	Head, Section on Biochemical Physiology	CM	NHLBI
	Joel Moss	Head, Section on Molecular Mechanisms	CM	NHLBI
	Adelbert A. Roscher	Guest Worker	CM	NHLBI
	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI

COOPERATING UNITS (if any)
Dr. Roscher is on a fellowship from the Max Kade Foundation, Inc., Austrian Academy of Sciences.

LAB/BRANCH
Cellular Metabolism

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.8	0.7

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Bradykinin increases production of prostaglandin E-2 and prostacyclin as measured by radioimmunoassay. Both of these prostaglandins as well as PGE stimulate cAMP production and accumulation in cultured human foreskin fibroblasts. Responsiveness to bradykinin increases as cells approach confluency. Maintenance of confluent fibroblast without replacement of serum-containing medium is accompanied by a loss in responsiveness to bradykinin as measured by both cAMP accumulation and prostaglandin production. Incubation with fresh medium containing serum for at least 3 h restores responsiveness to bradykinin. The increment in cAMP produced by bradykinin is reduced when cells are incubated with isoproterenol, 8 Br-cAMP, phosphodiesterase inhibitors, and forskolin, agents which themselves increase cell cAMP content. These agents also inhibit the production of prostaglandins stimulated by bradykinin, suggesting that elevations in cAMP content may inhibit prostaglandin production.

Project Description:

Objectives: To elucidate mechanisms whereby hormones and other effectors influence cyclic nucleotide metabolism and prostaglandin production in cultured cells; to specifically study the mechanism of bradykinin on prostaglandin formation and subsequent stimulation of cAMP accumulation.

Methods Employed: Cultured cells grown under standard conditions; cAMP and prostaglandins assayed by radioimmunoassay.

Major Findings: Human fibroblasts respond to bradykinin with increases in production of prostaglandin PGI_2 and PGE_2 and in intracellular cAMP. Since PGI_2 and PGE_2 activate adenylate cyclase in the fibroblasts, it is believed that the effect of bradykinin on cAMP is secondary to production of these prostaglandins. The magnitude of bradykinin effects on both prostaglandin production and cAMP increased in parallel as the fibroblasts approached confluency. In contrast, the fibroblasts responded similarly to exogenous prostaglandins whether rapidly growing or confluent. When confluent fibroblasts were maintained in serum-containing growth medium for 14 days without replacement with fresh growth medium, responsiveness to bradykinin decreased. The loss in responsiveness was partially reversed by incubation of nonresponsive fibroblasts for 3 h with fresh serum-containing growth medium. Cycloheximide did not prevent the restoration of responsiveness to bradykinin when either prostaglandin production or cAMP content was measured. Incubation of fibroblasts with serum did not alter responsiveness to prostaglandins.

Indomethacin (1 μM, 5 min) and dexamethasone (1 μM, 48 h) inhibited the effects of bradykinin on both prostaglandin production and intracellular cAMP; indomethacin and dexamethasone did not, however, affect the response of the cells to exogenous prostaglandins. These data are consistent with the hypothesis that the magnitude of cAMP accumulation in response to bradykinin may be limited by the capacity of the fibroblast to increase prostaglandin production when exposed to the kinin rather than by their ability to respond to prostaglandins.

Incubation of fibroblasts with bradykinin produced a transient increase in cAMP content which reached a maximum within several minutes and then declined. Intracellular cAMP determined in the presence or absence of bradykinin was increased by 1-isobutyl-3-methylxanthine or other phosphodiesterase inhibitors; the relative effect of bradykinin on cAMP content was, however, decreased in the presence of phosphodiesterase inhibitors. Prostacyclin and PGE_2 production by kinin-stimulated fibroblasts was also reduced when the fibroblasts were incubated with 8 Br-cAMP, isoproterenol, a β-adrenergic agonist, or forskolin, a diterpene which markedly increases intracellular cAMP. These data are consistent with the hypothesis that elevation in intracellular cAMP mediated by bradykinin may decrease bradykinin-stimulated prostaglandin production. Thus, through feedback inhibition of prostaglandin synthesis by cAMP, fibroblasts may modulate their own responsiveness to bradykinin.

[^{3}H]bradykinin ([^{3}H]BK)-binding sites have been identified and characterized in intact fibroblasts. At 4°C binding is rapid, equilibrium being reached by 30 min. Scatchard plots are linear (K^D 4-6 nM) with no suggestion of cooperativity

among binding sites. Different BK analogues compete for binding sites with [^{3}H]BK with varying potency. The potency of the analogues to compete with [^{3}H]-BK correlates with their potency to induce PGI formation. [^{3}H]BK-binding sites can be down regulated by preincubation of cells with BK at 37°C.

At 37°C, [^{3}H]BK is rapidly degraded and rapidly translocated to a cellular pool (compartment) that is resistant to extraction from cells with acid. Bacitracin inhibits degradation of bradykinin.

In preliminary experiments, we have found that incubation of intact fibroblast with bradykinin alters metabolism of phosphatidylinositol and phosphatidylcholine. Additional experiments are in progress to attempt to elucidate the mechanism of the bradykinin activation of phospholipase(s).

Significance to Biomedical Research: Bradykinin, a potent stimulus for prostaglandin production in many cell types, is thought to play an important role in maintenance of vascular tone and permeability and in inflammatory processes. Fibroblasts may constitute a good model system in which to study initial events in bradykinin action and prostaglandin metabolism.

Fibroblasts also constitute a good model system to study the coordinate and independent regulation of the responsiveness of the cyclase system to bradykinin, hormones, and other effectors.

Proposed Course: Continued study on the mechanism of action of bradykinin with emphasis on characterization of the bradykinin receptor and bradykinin activation of the arachidonic acid cascade.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00607-09 CM

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Cyclic GMP Metabolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Su-Chen Tsai	Research Chemist	CM	NHLBI
	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI
OTHER:	Vincent C. Manganiello	Head, Section on Biochemical Physiology	CM	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Cellular Metabolism

SECTION
Metabolic Regulation

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.2	1.2	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Activation of guanylate cyclase by nitrosyl hemoglobin (NOHb) or activator with MgCl-2 or MnCl-2 was investigated. Maximal enzyme was observed with NOHb and MgCl-2, MnCl-2, and activator or MnCl-2, activator, and NOHb. Excess of Mg-2+ or Mn-2+ above substrate concentration was required for optimal activity. NOHb and activator both did not alter kinetic constants of MgGTP, linear kinetic relation, or MnGTP, a negative cooperative nature. Activation by both compounds was inhibited by methylene blue and 1,10-phenanthroline. Inactivation of cyclase at 0°C was prevented by Hb, phenyl-Sepharose, methylene blue, or methyl viologen. Three monoclonal antibodies have been studied, only one of which appears to inhibit guanylate cyclase.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: To characterize guanylate cyclase and elucidate the mechanisms that regulate its activity.

Methods Employed: Guanylate cycalse is assayed in a system containing 1 mM (α-^{32}P)GTP, 5 mM cGMP, 6 mM theophylline, 10 µg of activator and Tris-HCl buffer, pH 7.4. Guanylate cyclase from rat liver supernatant is purified using salt fractionation, ion exchange, gel filtration, GTP affinity chromatography and electrophoresis to yield a homogeneous enzyme. Enzyme from GTP affinity chromatography, step VI, was used for most experiments.

To assay hybridoma medium for antibodies to guanylate cyclase, 50 µl of guanylate cyclase solution (step VI, 3 to 12 µg/ml) was transferred to each well of microtiter plates. After 2 to 6 h at 2°C, wells were washed with 100 µl of phosphate-buffered saline (PBS). A solution of 1% bovine serum albumin (100 µl) was added to each well and plates were stored at -20°C or -60°C. For assays, plates were washed with PBS and 25 µl of medium was added. After 3 h at room temperature, plates were washed with PBS, buffer containing 20% fetal calf serum and 10,000 cpm of ^{125}I-FAB was added to each well and incubation was continued for 1 h at 37°C.

Major Findings: Preparation of Monoclonal Antibodies to Rat Liver Guanylate Cyclase. Fusion of spleen cells from immunized mice with myeloma cells has yielded nearly 2% of hybrids that apparently produced antibodies to the soluble hepatic guanylate cyclase. Three hybridomas were further characterized after being cloned and recloned in soft agar. All produced antibodies of the IgM class; only one antibody appeared to inhibit catalytic activity.

Chacterization of Purified Hepatic Guanylate Cyclase. Guanylate cyclase from step VI or homogenous enzyme from step VII of purification was no longer activated by nitric oxide, but it was stimulated 30- to 100-fold by 1 µM nitrosyl hemoglobin (NOHb) in $MgCl_2$; activation was only 5- to 10-fold in $MnCl_2$.

The enzyme was very unstable at 0°C but in the presence of activator or 1 µM hemoglobin (Hb) remained active during 3 h incubation at 0°C; these additions also increased activity ~ 500 and 200%, respectively. In the presence of NOHb, activity decayed with a half-time of < 30 min at 0°C; re-addition of "NO" did not restore activity.

Usually enzyme activity was greater with $MgCl_2$ than it was in the presence of $MnCl_2$ and activator ± NOHb. However, activation by NOHb varied a great deal, perhaps because of variations in the molecular species present in the NOHb preparations, i.e., $(Hb)_4$, $(Hb)_4NO$, $(Hb)_4(NO)_2$, $(Hb)_4(NO)_3$, and $(Hb)_4(NO)_4$. With concentrations of NOHb above optimal, activation was decreased. Optimal pH for maximal activation by NOHb was 7.2 to 7.7, similar to that observed in its absence or with activator. Excess of $MgCl_2$ or $MnCl_2$ above GTP was required for guanylate cyclase activity (basal or NOHb-activated). With Mg^{2+}-GTP plots of 1/v vs 1/s were linear (K_m ~ 0.35 mM) and with Mn^{2+}-GTP could be described as indicative of negative cooperativity with apparent K_m's of ~ 1 mM and 30-60 µl. These kinetic characteristics were not altered by NOHb or activator or changes in pH between 6.5 and 8.5.

Activation of guanylate cyclase by NOHb, protoporphyrin, or activator was inhibited by methylene blue; 1,10-phenanthroline inhibited activation by NOHb or activator but not by protoporphyrin. Rotenone inhibited 40-50% with NOHb or activator; it completely prevented activation by protoporphyrin. NAD, NADP, FMN, or FAD inhibited partially stimulation by NOHb or protoporphyrin but not by activator. Thus, although activation of guanylate cyclase by activator, NOHb, or protoporphyrin IX apparently proceeded through an oxidation-reduction process, it seemed that the mechanisms of action of these compounds might be different.

Basal guanylate cyclase activity was increased in $MgCl_2$ or $MnCl_2$ by NAD, NADP, FAD, FMN, mersalyl-(DTT added), methyl viologen, methylene blue, Hb, hemin, 1,10-phenanthroline, and phenyl-Sepharose gel. Methyl viologen, methylene blue, hemoglobin and phenyl-Sepharose also stabilized enzyme activity at 0°C but NAD, NADH, FMN and FAD did not. Guanylate cyclase bound to phenyl-Sepharose was no longer activated by Hb or activator, but it was activated by NOHb or protoporphyrin. It appears that certain materials, e.g., Hb, phenyl-Sepharose, because of their hydrophobic properties may enhance guanylate cyclase activity and stability.

Significance to Biomedical Research: Although at present information concerning the metabolism and functions of cyclic GMP is limited, it appears that this nucleotide may be of special importance in the development, physiology, and pathology of lung, vascular smooth muscle, and kidney.

Proposed Course: We shall further characterize the regulatory properties of purified guanylate cyclase and define the molecular mechanism of activation by NOHb and related compounds. Monoclonal antibodies will be used in some of this work as well as for studies of factors that may control the amount of enzyme protein in cells.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00614-05 CM

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Calmodulin-Activated Phosphodiesterase: Interaction of Calmodulin with Phosphodiesterase and other Binding Proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Randall L. Kincaid	Staff Fellow	CM	NHLBI
	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI

COOPERATING UNITS (if any)

Dr. James C. Osborne, Jr., NHLBI, NIH.

LAB/BRANCH
Cellular Metabolism

SECTION
Metabolic Regulation

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Calmodulin (CaM)-dependent cyclic nucleotide phosphodiesterase (PDE) prepared from ovine and bovine brain display different maximal activities, amino acid compositions, and proteolytic fragments. An improved affinity chromatography purification procedure using pyridyldithiopropionyl CaM linked to thiol-Sepharose results in reproducible, quantitative elution of PDE activity. Dansyl-CaM, an active fluorescent derivative, exhibits two classes of Ca-2+-binding sites; occupation of two higher affinity sites (Kd ~ 1 μM) corresponds to an increase in fluorescence and appears to involve cooperative Ca-2+ binding, whereas Ca-2+ binding to two lower affinity sites (Kd ~ 20-50 μM) parallels a decrease in fluorescence. The apparent affinity for Ca-2+ was increased at low NaCl concentrations while the decrease in fluorescence intensity was not seen at elevated $MgCl_2$ concentrations, suggesting Mg-2+ competition for Ca-2+ sites. Interaction of dansyl-CaM with several binding proteins required occupation of the two high affinity Ca-2+ sites; their affinity was not altered in the presence of binding protein. These data suggest three distinct solution conformers of CaM, one of which [CaM.2(Ca-2+)] is sufficient for complex formation with PDE and several other CaM-binding proteins.

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Project Description:

Objectives: To characterize and compare the physical and enzymatic properties of purified calmodulin-dependent phosphodiesterase (PDE) from bovine and ovine brain. To define the functional relationship between Ca^{2+} binding to calmodulin (CaM), its interaction with several binding proteins, and activation of enzyme activity.

Methods Employed: Purification of PDE was carried out essentially as described last year. In some experiments, a new CaM affinity purification step using disulfide-linked CaM-Sepharose was employed (see Major Findings). Calcineurin, a major CaM-binding protein in brain, was purified to homogeneity by elution from DEAE-BioGel A using an increase in ionic strength (at pH 5.5) followed by successive chromatography on Cibacron Blue-Sepharose, CaM-Sepharose, and gel filtration using high performance liquid chromatography. Homogenous troponin I and myosin light chain kinase were gifts of Dr. Robert Adelstein, NHLBI.

Dansyl-CaM was prepared as described last year. Measurements of fluorescence intensity and polarization of fluorescence were made after addition of Ca^{2+} and/or other metal ions in the presence or absence of proteins that interact with CaM.

Pyridyldithioproprionyl CaM (PDP-CaM) was prepared by incubation of N-(succinimidyl) 2-pyridyldithioproprionate and CaM at pH 7.5 followed by exhaustive dialysis at pH 5.3. Disulfide-linked CaM-Sepharose (~ 4 mg CaM/ml gel) was prepared by incubation of PDP-CaM with the reduced form of activated thiol-Sepharose 4B at pH 7.4 followed by repeated washing of the gel. Limited proteolysis using α-chymotrypsin (0.7 µg/ml) was carried out at 30°C with purified PDE (20 µg/ml) in Tris·HCl buffer, pH 8.0, containing 0.25 M NaCl, 0.1 mM EGTA and 5 mM $MgCl_2$.

Major Findings: Extensive characterization of PDE from bovine and ovine brain indicated that they have different physical and kinetic properties. The specific activity of purified enzyme from bovine brain was approximately 30% greatear than that from ovine brain; half-maximal activation (1-2 nM) and degree of activation (8- to 12-fold) by CaM were, however, the same. The amino acid composition of the enzymes from the two species was quite similar with the exception that proline appeared to be absent from the ovine enzyme. Finally, in collaboration with Dr. Stith-Coleman, it was shown that the sodium dodecyl sulfate peptides resulting from limited proteolysis with α-chymotrypsin were different for the two species, being 57K and 45K daltons for bovine and 55K, 53K, and 38K daltons for ovine enzyme. The final proteolytic products, as shown previously for the bovine enzyme, were fully active in the absence of CaM.

Affinity chromatography utilizing disulfide-linked CaM-Sepharose was used for purification of PDE and resulted in increased yields of 11-15% for bovine and ovine enzyme. After coupling PDP-CaM (~ 2.8 mol pyridyldithioproprionate/mol CaM) to the reduced form of thiol-Sepharose, the affinity gel was used to bind PDE and other CaM-binding proteins. After washing, the bound proteins and calmodulin were quantitatively desorbed in the presence of dithiothreitol and separated by gel filtration. Recovery of activity (> 80%) was entirely

reproducible, since reuse of the affinity gel was not necessary. The binding capacity of the affinity gel for PDE was ~ five times greater than for CNBr-prepared gels of similar CaM substitution. This general procedure may prove useful in other affinity chromatography systems, where recovery of matrix-bound proteins is difficult to achieve.

Preparations of dansyl-CaM (0.5-0.6 mol dansyl/mol CaM) appeared identical to native CaM in the analytical ultracentrifuge and in their ability to activate phosphodiesterase activity. After cleavage with CNBr, greater than 85% of the dansyl fluorescence was found in one peptide when analyzed by anion-exchange high performance liquid chromatography, indicating modification of predominantly one domain in CaM.

The Ca^{2+}-dependent changes in the fluorescence of dansyl-CaM were markedly influenced by the concentration of $MgCl_2$ and NaCl. At Mg^{2+} concentrations below 1 mM, increasing Ca^{2+} concentrations produce a sharp increase in fluorescence (apparently $K_{1/2}$ ~ 1 μM) suggestive of positively cooperative calcium binding followed by a gradual decrease in fluorescence intensity to approximately 60-70% of the peak intensity. The decrease in intensity was not observed at Mg^{2+} concentrations of 5 mM or greater, suggesting that Mg^{2+} can compete for Ca^{2+}-binding sites. The increase in fluorescence intensity occurred at lower free Ca^{2+} concentrations with NaCl ≤ 50 mM, suggesting that reduced ionic strength increased the affinity of the Ca^{2+}-binding sites.

Titration of Ca^{2+} site occupancy with 20 to 40 μM dansyl-CaM indicated that the maximal increase in fluorescence intensity occurred when the first two sites were occupied and that the decrease in fluorescence observed at low Mg^{2+} concentrations corresponded to filling the third (and fourth) site(s). In collaboration with Dr. J. Osborne, parallel studies of the circular dichroism of CaM indicate that conformational events occur concomitant with these changes in fluorescence; these data suggest that at least three distinct solution conformers of CaM exist, depending on the number of Ca^{2+} sites occupied.

Quantitative studies of dansyl-CaM interaction with troponin I, calcineurin, PDE, and myosin light chain kinase indicated that the additional fluorescence increase produced by the binding protein (see Z01 HL 00614-04 CM) in the presence of 100 μM Ca^{2+} was proportional to the amount of binding protein and maximal at a stoichiometry of 1 mol binding protein:1 mol dansyl-CaM. Measurements of polarization of fluorescence indicated that complex formation accompanied the "cooperative" binding of Ca^{2+} to the two high affinity sites on dansyl-CaM and that changes in fluorescence intensity paralleled those in polarization. Furthermore, the Ca^{2+} dependence of the increase of dansyl fluorescence was not markedly altered by the presence of saturating amounts of any binding protein, indicating that the affinity of the high affinity sites was not increased. Thus, it appears that complex formation with a binding protein occurs when Ca^{2+} is bound at the two high affinity sites on CaM.

Significance to Biomedical Research: The effects of many biochemical regulators, such as hormones, on mammalian cells are mediated by altering the rates of synthesis and/or degradation of cyclic nucleotides. The understanding of the properties of the enzyme regulating cyclic nucleotide degradation is important for the understanding of normal and pathological cellular activity and may permit

design of rational therapeutic approaches. The well-established significance of calmodulin in control of diverse cellular functions makes the elucidation of its mechanism of action important for the understanding of Ca^{2+}-regulated events.

Proposed Course: With the purified phosphodiesterase continued structure-function studies are planned to elucidate the molecular basis of its activation by CaM, by phospholipids and by limited proteolysis. To establish whether the interaction of a binding protein and CaM with two moles of bound Ca^{2+} is sufficient to alter function of the binding protein, an assay is being developed that will permit measurements of phosphodiesterase activity under exactly the same conditions used for the physical assessment of interaction, and it may be possible to carry out analogous experiments with other CaM-binding proteins.

Publications:

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Kincaid, R.L., Vaughan, M., Osborne, J.C., Jr., and Tkachuk, V.A.: Ca^{2+}-dependent interaction of dansyl-calmodulin with cyclic nucleotide phosphodiesterase, calcineurin, and troponin I. J. Biol. Chem., in press, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00622-05 CM

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Regulation of Cyclic Nucleotide Metabolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Joel Moss	Head, Section on Molecular Mechanisms	CM	NHLBI
	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI

COOPERATING UNITS (if any)
Dr. James C. Osborne, Jr., NHLBI, NIH; Dr. Peter H. Fishman, NINCDS, NIH; Dr. Donald C. Robertson, Univ. of Kansas, Lawrence, KS 60645.

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Cellular Metabolism

SECTION
Molecular Mechanisms

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	0.6	1.0

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1) Following a brief exposure of cultured cells to 125-I-choleragen, adenylate cyclase remained persistently activated. The affinity of 125-I-toxin for intact cells obtained by kinetic analysis was similar to that of free toxin for GM1-oligosaccharide. Total 125-I-choleragen incorporated into cells was degraded with a half-life of greater than 24 hr. The slow degradation of the toxin may explain the continued activation of cyclase following a brief exposure of cells to toxin. 2) Interaction of Escherichia coli heat-labile enterotoxin (LT) with GM1 and its oligosacccharide was similar to that observed with choleragen; both toxins may share similar ganglioside receptors. In contrast to choleragen, the ADP-ribosyltransferase activity of LT was not fully expressed without prior trypsinization to activate the catalytic subunit. 3) A histone-dependent NAD glycohydrolase was identified in rat liver; histone was required for hydrolysis of NAD and for the pyridine base exchange reaction. A histone-dependent NAD: arginine ADP-ribosyltransferase was converted from an active high molecular weight species to an active protomeric form by histone; a similar transition was not observed with the glycohydrolase. Activation of the ADP-ribosyltransferase and the NAD glycohydrolase by histone was rapid and reversible.

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PHS-6040
(Rev. 2-81)

Project Description:

Objectives: To study the regulation of cyclic nucleotide metabolism. The adenylate cyclase system is responsible for the synthesis of cyclic AMP. Studies on the activation of adenylate cyclase by choleragen (cholera toxin) defined the G/F component of the cyclase system. Studies of analogies to the choleragen-catalyzed reaction in animal cells led to the identification of NAD: arginine ADP-ribosyltransferases in human and avian erythrocytes. In the present report, we have used the choleragen-fibroblast system to develop a kinetic model for the interaction of cells with ligands and to follow the fate of ligands bound to receptors. The model appears to be general and applicable to the study of the hormone and drug interaction with their target tissues. Investigations of the localization of the NAD:arginine ADP-ribosyltransferase led to the identification of a new enzyme, which catalyzes the histone-dependent hydrolysis of NAD and the pyridine base-exchange reaction. The properties of this protein were investigated.

Methods Employed: Assays. ADP-ribosyltransferase and NAD glycohydrolase activities were determined by our published methods. The turkey erythrocyte ADR ribosyltransferase, purified by our published procedure, showed one major band on sodium dodecyl sulfate-polyacrylamide gels.

Major Findings: Degradation of Choleragen Bound to Cultured Cells. Choleragen (cholera toxin) appears to exert its effects on cells through activation of adenylate cyclase. The initial step in toxin action is its binding through its B subunit to a cell surface receptor, believed to be ganglioside G_{M1}. Subsequently, the A_1 peptide activates adenylate cyclase by catalyzing the NAD-dependent ADP-ribosylation of an arginine or arginine-like residue in a critical regulatory subunit of the adenylate cyclase system.

The mechanism by which the A_1 peptide associates with the cyclase is not clear. To understand the fate of cell-bound toxin and to try to explain the persistence of toxin effects on cells, we have monitored the fate of ^{125}I-choleragen bound to human fibroblasts and mouse neuroblastoma cells.

^{125}I-choleragen bound to human fibroblasts was degraded slowly with a $t_{1/2}$ of 1-2 days; the radiolabel in bound ^{125}I-choleragen was present in both the A and B subunits. During degradation, radiolabel was lost more rapidly from the ^{125}I-A_1 ($t_{1/2}$ 1 day) than from the ^{125}I-B peptides ($t_{1/2}$ > 5 days). ^{125}I-choleragen bound to neuroblastoma cells showed a considerably shorter $t_{1/2}$ for both the ^{125}I-A_1 and ^{125}I-B peptides; as with the fibroblasts, radiolabel was lost more rapidly from the ^{125}I-A_1 than from the ^{125}I-B peptides. The continued presence of choleragen in the fibroblasts and neuroblastoma cells was associated with a prolonged activation of adenylate cyclase. In addition, fibroblasts, previously exposed to toxin and then washed free of unbound choleragen, only slowly recovered their ability to bind ^{125}I-choleragen with a $t_{1/2}$ of 7 days. Fibroblasts exposed to choleragen also showed evidence of persistent toxin on the surface based on the ability of the cells to bind antitoxin, antisubunit A, or antisubunit B antibodies followed by ^{3}H-protein A. It appears that choleragen remains persistently bound to fibroblasts, is degraded at a slow rate, and may prevent the binding of new toxin molecules to the fibroblast. The relatively slow degradation of toxin by fibroblasts may explain the prolonged

activation of adenylate cyclase by toxin. The loss of ^{125}I-toxin binding following incubation with toxin may result from continued presence of toxin subunits on the cell surface.

Analysis of Irreversible Binding of Choleragen to Cells. The initial event in the action of many agonists is the binding of the agent to cell surface receptors. Cellular response may be a direct result of formation of the ligand-receptor complex or require processing of the complex; processing may occur within the plane of the plasma membrane and, in some instances, result in internalization of the agonist. Processing of the agonist removes some of the cell-associated agent from direct and freely reversible interaction with free agonist in the medium. Due to the processing of the agonist, and the nonequilibrium state of the cell-associated agonist with that free in the medium, analysis of the total cell-associated agonist for determination of receptor sites by methods dependent on equilibrium of free agonist with that associated with the cell is no longer justified. Although the individual events involved in processing may, in fact, be reversible, they result in removal of a fraction of cell-associated agonist from direct contact with agonist in the medium and in a slowly reversible system may lead to a nonequilibrium situation. Current studies on receptor-ligand interactions usually employ a "binding" step followed by extensive washing to remove free ligand. This procedure, by definition, removes reversibly bound ligand from the receptor; the equations used by most workers to analyze the data, however, require an equilibrium between free and bound ligand and are not applicable given the design of most binding assays. The assay, in fact, measures binding that is slowly reversible or irreversible. Assuming that ligand-receptor interaction involves two stages with a reversible step followed by an "irreversible" event, the K_a for the reversible reaction may be obtained from the rate of the "irreversible" step. ^{125}I-choleragen binding to human fibroblasts was only slowly reversible at both 0°C and 37°C. The interaction of choleragen (cholera toxin) with human fibroblasts appears therefore to be an example of "irreversible" binding. The two-step model was therefore applied experimentally to determine the K_a for the reversible step in ^{125}I-choleragen binding to human fibroblasts at 0°C ($K_a = 1.9 \times 10^8\ M^{-1}$) and at 37°C ($K_a = 3.6 \times 10^8\ M^{-1}$). As predicted by the two-step model for ligand binding, the addition of 50 µg/ml of unlabeled toxin enhanced the rate of release of radioactivity at 37°C; the rate of radiolabel release remained low at 0°C, even with unlabeled toxin present in the medium. The rate of release of previously incorporated ^{125}I-toxin was accelerated by toxin (50 µg/ml > 5 µg/ml). The two-step model for ligand binding appears to be applicable to the study of ^{125}I-choleragen binding to fibroblasts and should be useful, in general, for the analysis of receptor-ligand interaction.

Ganglioside Specificity and Transferase Activity of Escherichia coli Heat-Labile Enterotoxin. *E. coli* heat-labile enterotixn (LT) appears to be functionally, structually, and immunologically similar to choleragen. Both toxins consist of A and B components. The B component of choleragen probably consists of five identical subunits and binds to specific receptors on the cell surface. The receptor is believed to be the ganglioside G_{M1}, and the specificity for the interaction of choleragen with ganglioside appears to reside with the B component of choleragen and the oligosaccharide of G_{M1}. Although G_{M1} enhances the responsiveness of G_{M1}-deficient cells to LT, there is conflicting evidence as to whether G_{M1} is the receptor for LT.

Both toxins possess NAD glycohydrolase and ADP-ribosyltransferase activities, and it is believed that LT, like choleragen, activates adenylate cyclase through an NAD-dependent ADP-ribosylation of a critical regulatory component of the cyclase system. Differing effects of the two toxins on the cells have been observed, however. This could be explained if the catalytic activity of the A component of LT were expressed under conditions different from those required for choleragen. The availability of homogenous LT permitted us to study the interaction of LT and its components with gangliosides and their corresponding oligosaccharides, to examine the requirements for expresssion of ADP-ribosyltransferase activity, and to compare these properties of LT with those of choleragen.

Escherichia coli heat-labile enterotoxin (LT) bound to rat glioma C6 cells that had incorporated ganglioside G_{M1} but not to cells that had taken up gangliosides G_{M2}, G_{D1a}, oir G_{D1b}. The same specificity also was observed with choleragen, the enterotoxin of Vibrio cholerae. The tryptophanyl fluorescence spectra of LT and its B component differed from those of choleragen and its B component, respectively. The oligosaccharide moiety of G_{M1} "blue-shifted" the tryptophanyl fluorescence of LT and its B component, whereas neuramin lactose and the oligosaccharide of G_{D1a} did not. As reported previously (Fishman, P. H., Moss, J., and Osborne, J.C., Jr.: Biochemistry 17, 711-716, 1978), G_{M1}-oligosaccharide also "blue-shifted" the fluorescence spectra of choleragen at concentrations similar to those observed to be effective with LT.

LT exhibited NAD glycohydrolase and ADP-ribosyltransferase activities, which were stimulated by dithiothreitol. Incubation of LT, but not choleragen, with trypsin increased the ADP-ribosyltransferase activity of LT over 200%. The specific enzymatic activity of trypsin-treated LT was similar to that of choleragen. The enzymatically active A component of LT is a single polypeptide chain, whereas the A component of choleragen consists of two polypeptide chains linked together by a disulfide bridge. It would appear that trypsin cleaves the A component of LT and generates a fragment with enhanced ADP-ribosyltransferase activity.

Both LT and choleragen have the same specificity for G_{M1} and its oligosaccharide and may share similar receptors. The two toxins, however, differ in that the catalytic activity of LT is not fully expressed without prior trypsinization. This may contribute to differences in the toxicity of LT and choleragen for different types of cells.

Activation of a Rat Liver NAD Glycohydrolase by Histone. NAD is a substrate for two distinct types of enzymatic reactions: oxidation-reduction and ADP-ribose transfer. In the latter, NAD functions as an ADP-ribose donor with the ADP-ribose acceptor dependent on the specificity of the enzyme. Two apparently different types of enzymes exist, those that use water as an ADP-ribose acceptor (NAD glycohydrolases) and those that attach the ADP-ribose to a specific protein (ADP-ribosyltransferases). ADP-ribosyltransferases, in some systems, have been shown to have important regulatory roles; this function has been most clearly identified for the bacterial toxins choleragen and diphtheria toxin, which activate adenylate cyclase and inhibit protein synthesis, respectively. The role of the NAD glycohydrolase is not certain; the raison d'etre of an enzyme that cleaves NAD to ADP-ribose and nicotinamide is not immediately

apparent. The glycohydrolases may, in fact, be ADP-ribosyltransferases whose physiological substrates have not been identified; the glycohydrolase activity would in this case result from an abortive reaction. Indeed, ADP-ribosyltransferases can act as NAD glycohydrolases. Alternatively, an NAD glycohydrolase could regulate the levels of NAD or its cleavage products ADP-ribose and nicotinamide. For this control to be effective, we postulated that independent effectors msut exist which determine glycohydrolase activity and earlier observed that histone activates an ADP-ribosyltransferase that uses arginine, other guanidino compounds, and proteins as effectors. Others have shown that the poly(ADP-ribose) synthetase exists in histone-dependent and independent forms. It appeared reasonable that histone, in addition to controlling ADP-ribosylation, might also regulate NAD levels by effects on an NAD glycohydrolase.

An NAD glycohydrolase which was dependent on histones for hydrolysis of NAD to ADP-ribose and nicotinamide was partially purified from rat liver cytosol. The purification procedure sequentially utilized hydrophobic chromatography on phenyl-Sepharose, ion-exchange chromatography on both carboxymethyl cellulose and DEAE-cellulose, and gel permeation chromatography on Ultrogel AcA 44. On Ultrogel AcA 44, the histone-dependent enzyme eluted in the vicinity of bovine serum albumin (67,000 daltons). The purification procedure effectively resolved the histone-dependent NAD glycohydrolase form soluble histone-independent NAD glycohydrolases, primarily by hydrophobic chromatography on phenyl-Sepharose. In the presence of histone, the ratio of ADP-ribose release from [adenine-U-^{14}C]NAD to [carbonyl-^{14}C]nicotinamide release from [carbonyl-^{14}C]NAD was ~ 1.17. The enzyme possessed a broad pH optimum with a maximum in glycine HCl at 8.5; the K_m for [carbonyl-^{14}C]NAD was 18 μM. In the presence of [carbonyl-^{14}C]nicotinamide and NAD, the enzyme catalyzed the histone-dependent formation of [carbonyl-^{14}C]NAD. The histone-dependent NAD glycohydrolase thus catalyzes both NAD hydrolysis to ADP-ribose and nicotinamide (Reaction 1) and the pyridine base exchange reaction (Reaction 2).

(1) [carbonyl-^{14}C]NAD $\longrightarrow$ [carbonyl-^{14}C]nicotinamide + ADP-ribose + H^+

(2) [carbonyl-^{14}C]nicotinamide + NAD $\rightleftharpoons$ [carbonyl-^{14}C]NAD + nicotinamide

In contrast to the histone-dependent mono(ADP-ribosyl)transferase and poly(ADP-ribose) synthetase, the enzyme failed to form stable ADP-ribosyl linkage to guanidino compounds or histone using [adenine-U-^{14}C]NAD as the [adenine-U-^{14}C]ADP-ribose donor.

The effect of histones on the glycohydrolase appears to be relatively specific. Of the proteins examined, only histones were capable of activating the glycohydrolase; of note is the fact that polyarginine and polylysine were inactive. A role for histone or a similar effector has not been observed with other NAD glycohydrolases. Due to the almost absolute dependence of this enzyme on histone, it is apparent that enzymatic activity would not be identified under routine assay conditions. In fact, it appears that only a small fraction of soluble rat liver NAD glycohydrolase activity is dependent on histone. Based on this and prior reports, it appears that rat liver contains NAD glycohydrolases and (ADP-ribosyl)transferases which can exist in histone-dependent forms.

Activation of an ADP-ribosyltransferase by Histone. An NAD:arginine ADP-ribosyltransferase from turkey erythrocytes was shown previously to exist in histone-dependent and independent forms. The effect was specific in that the NAD:arginine ADP-ribosyltransferase activity of choleragen was not activated by histone.

With the erythrocyte enzyme, histone decreased the apparent K_m values for arginine methyl ester and agmatine and increased the resistance of the transferase to thermal denaturation. Activation of the transferase by histones was rapid, with a minimal delay observed upon addition of histones to a histone-free assay. Activation by histones was reversed upon dilution of a sample containing histones into an assay mix free of histone. In the absence of histone, the transferase existed as a rapidly sedimenting species; in the presence of histone, the transferase sedimented as a promoter.

Significance to Biomedical Research: The pulmonary and cardiovascular systems are affected under physiological and pathological conditions by extracellular agents such as hormones and toxins. The lung, in particular, is exposed through the tracheobronchial tree to a variety of bacterial and toxic agents. To be effective, some of these agents must interact with a cellular receptor, and a substantial number of these agents exert their effects by altering the steady-state levels and localizations of cyclic nucleotides within the cell. A number of pharmacological agents have been in use which override aberrant physiological control to the benefit of the patient; these agents have been designed to interact with specific cellular receptors. By using cultured cells, model systems and purified preparations, it may be possible to simplify and define the factors critical to cyclic nucleotide metabolism. These models can then be used to understand the controls which operate in the more complex pulmonary and cardiovascular system.

Proposed Course: 1) To define the mechanism of activation of adenylate cyclase by hormones and toxin. Choleragen will be used as a probe to define the interaction of agents such as hormones and drugs with surface receptors and to resolve the steps involved in internalization of the agent. 2) To identify the enzymes involved in ADP-ribosylation in animal cells and the possible regulatory role(s) of this covalent modification.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00625-04 CM

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

ADP-Ribosyltransferases: Characterization of their Substrates and Factors that Control their Activity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Paul A. Watkins	Research Associate	CM	NHLBI
	David A. Yost	Staff Fellow	CM	NHLBI
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	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Cellular Metabolism

SECTION

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NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.2	0.3

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Choleragen activates adenylate cyclase by catalyzing the ADP-ribosylation of guanyl nucleotide-binding regulatory components (G) of adenylate cyclase. The ADP-ribose linkage was stable for 30°C for 2 h with or without GTP, whereas GTP was required to stabilize the activity of G. A transferase purified from turkey erythrocytes catalyzed the NAD-dependent ADP-ribosylation of multiple soluble proteins from bovine thymus. Nucleoside triphosphates stimulated this process (ATP > ITP = GTP > CTP = UTP). It appears that ADP-ribosylation of cellular proteins by endogenous ADP-ribosyltransferases may be subject to regulation by nucleoside triphosphates. Diethyl pyrocarbonate rapidly inactivated the turkey transferase at 0-4°C. Conditions known to activate the enzyme almost doubled the rate constant for inactivation. Enzyme inactivation was dependent on pH. Protection from diethyl pyrocarbonate inactivation could only be achieved in the presence of a complete dinucleotide. Nicotinamide, ADP-ribose, or arginine, or arginine-like compounds did not alter the rate constant for inactivation. A new NAD:arginine ADP-ribosyltransferase was purified from turkey erythrocytes. This enzyme, unlike the transferase previously purified from turkey erythrocytes, was unaffected by chaotropic salts or histone.

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PHS-6040
(Rev. 2-81)

Project Description:

Objectives: Choleragen activates adenylate cyclase by catalyzing the ADP-ribosylation of a guanyl nucleotide-binding regulatory component of the system; GTP is required for both activation and ADP-ribosylation. GTP is also required to maintain choleragen-activated bovine brain cyclase in the active state following toxin removal. To determine the correlation between cyclase inactivation, loss of ADP-ribose, and the GTP requirement, two cells with well-characterized choleragen substrates were studied: human skin fibroblasts and erythrocytes. Information obtained by use of these model systems should provide insight into requirements for hormonal control of adenylate cyclase. Like choleragen, a transferase purified from turkey erythrocytes catalyzes the ADP-ribosylation of many proteins; to probe the similarities and differences between these two enzymes, the effects of GTP and other nucleoside triphosphates on the erythrocyte transferases were studied. It is important to establish the role of other transferases in cell function. Our present investigations of these enzyme activities have defined important information on the regulation of these enzymes and also have demonstrated the existence of multiple forms of ADP-ribosyltransferase activity.

Methods Employed: Human skin fibroblasts or erythrocyte membranes were incubated with NAD (or [^{32}P]NAD) and activated choleragen, with or without GTP; washed membranes were then incubated further with or without GTP before assay of adenylate cyclase activity or SDS-polyacrylamide gel electrophoresis and radioautography.

Purified proteins or fractions from bovine thymus were incubated with purified turkey erythrocyte transferase and [^{32}P]NAD; proteins were precipitated with trichloroacetic acid and either collected on filters for radioassay or subjected to electrophoresis and radioautography.

Enzyme Assays. NAD:arginine ADP-ribosyltransferase activity was determined by published procedures. Product identification was achieved by the use of high pressure liquid chromatography.

Enzyme Purification. Turkey erythrocyte transferase A was purified by published procedures. Transferase B was purified by chromatography or phenyl-Sepharose, concanavalin A-Sepharose, carboxymethyl cellulose, Procion Red Agarose, and finally gel filtration or AcA 54.

Major Findings: Human Fibroblasts: Effect of GTP on Stability of ADP-Ribosyl-G. Incubation of fibroblast membranes with choleragen under conditions previously shown to ADP-ribosylate specifically peptides of 42,000 and 47,000 daltons resulted in a sixfold activation of adenylate cyclase. On incubation of the activated cyclase at 30°C without GTP, there was a rapid loss of activity; when GTP was present, cyclase activity was stable for at least 1 h. This GTP effect is similar to that found previously in this laboratory for brain adenylate cyclase. When fibroblast particulate proteins, ^{32}P-ADP-ribosylated by choleragen, were incubated for 1 h at 30°C, however, no loss of radioactivity from the 42,000 or 47,000 dalton proteins was observed whether or not GTP was present. The decrease in cyclase activity, therefore, did not result from the de-ADP-ribosylation of these proteins.

Human Erythrocytes: Effect of GTP on Stability of ADP-Ribosyl-G. Since fibroblasts contain the catalytic subunit (C) of adenylate cyclase as well as G, it was not clear whether the stability of the ADP-ribosylated G and/or stabilization of the choleragen-activated cyclase by GTP required the presence of C. To investigate this possibility, human erythrocyte membranes, which contain G but lack C, were used. When activated membranes were solubilized and then reconstituted with cyc⁻ membranes (which contain C but lack G), cyclase activity was 11 times that of cyc⁻ membranes reconstituted with extracts of erythrocyte membranes not activated with choleragen. Incubation of choleragen-activated erythrocyte membranes at 30°C without GTP resulted in a loss of the capacity to reconstitute cyclase activity, which was partially prevented when GTP was present during the 1-h incubation. Radioactivity in the 42,000 dalton erythrocyte peptide, ^{32}P-ADP-ribosylated by choleragen under identical conditions, was stable at 30°C whether or not GTP was present, despite the decrease in ability to reconstitute cyclase activity. Thus, it appears that the stability of ADP-ribosyl-G requires neither C nor GTP.

In erythrocyte membranes, several proteins in addition to the 42,000 dalton protein were ^{32}P-ADP-ribosylated. Certain of these, notably peptides of 50,000 and 87,000 daltons, lost radioactivity during incubation of membranes at 30°C. There is no evidence that either of these peptides is associated with erythrocyte G, and the loss of label was not detectably influenced by GTP. Thus, although ADP-ribosyl moieties on some proteins modified by choleragen may be cleaved by endogenous enzymes, perhaps phosphodiesterases, which are widespread in animal tissues, others, e.g., those on G, are seemingly less susceptible to degradation or removal. Whatever the reason for the stability of the ADP-ribosyl groups on G, it may explain the apparently irreversible nature of choleragen activation of adenylate cyclase in many types of cells.

Erythrocyte Transferase: Effect of Nucleoside Triphosphates. The choleragen-catalyzed ADP-ribosylation of many proteins was previously shown to be enhanced by nucleoside triphosphates, particularly GTP. Like choleragen, the erythrocyte transferase reaction was enhanced by nucleoside triphosphates when either a crude protein mixture (20,000 x g supernatant from bovine thymus) or purified proteins (e.g., lysozyme) served as substrate. In contrast to the choleragen-catalyzed reaction, that catalyzed by erythrocyte transferase was enhanced most effectively by ATP when either thymus proteins or lysozyme were substrate. GTP and ITP were somewhat less effective than ATP; CTP and UTP were less effective than the purine nucleotides. Half-maximal stimulation of ADP-ribosylation was observed with 8 mM ATP when the thymus protein fraction served as substrate and with 2.5 mM ATP when lysozyme was the substrate. ATP increased ADP-ribosylation of both thymus proteins and lysozyme in the presence of 20 to 200 μM NAD; with 2 mM NAD, ATP had no effect and with 10 mM NAD, ATP decreased labeling of lysozyme.

The nucleotide effect on transferase-catalyzed ADP-ribosylation was specific for the triphosphate ATP; ADP, AMP, cAMP, and inorganic pyrophosphate were not effective. App(NH)p, a nonhydrolyzable analog of ATP, was half as effective as ATP, but the corresponding guanosine derivative, Gpp(NH)p, was ineffective. Inorganic tri- and tetrapolyphosphate were also half as effective as ATP. Increasing concentrations of NaCl up to 100 mM did not enhance ADP-ribosylation; however, the magnitude of the ATP effect was decreased in the presence of 100 mM NaCl.

A number of purified proteins were ADP-ribosylated to different degrees by the transferase. ATP enhanced ADP-ribosylation of β-globulin, DNase I, and trypsin inhibitor (as well as lysozyme), decreased ADP-ribosylation of several others (e.g., bovine plasma albumin, polyarginine), and had no effect on others (e.g., histone f_{2a}, α-globulin). With both choleragen and the erythrocyte transferase, the effects of nucleotides on ADP-ribosylation were dependent on the protein substrate. Lysozyme was ADP-ribosylated by both enzymes, but enhancement by nucleoside triphosphate was observed only with the erythrocyte transferase; the choleragen-catalyzed reaction was unaffected by either GTP or ATP. Conversely, ADP-ribosylation of histone f_{2a} by the transferase was unaffected by ATP and inhibited by GTP, whereas the choleragen-catalyzed reaction was enhanced by either ATP or GTP. Thus, the responses of choleragen and the turkey erythrocyte transferase to nucleoside triphosphates differ as do their substrate specificites.

Selective Modification of the Active Protomeric Form of NAD:Arginine ADP-Ribosyltransferase by Diethyl Pyrocarbonate. Turkey erythrocyte NAD:arginine ADP-ribosyltransferase was rapidly inactivated by diethyl pyrocarbonate (DEP) at 0°C. A linear relationship was observed between the pseudo first-order rate constant for ADP-ribosyltransferase activation and DEP concentration. A second-order rate constant of 4.0 min^{-1} M^{-1} at 0°C was calculated. Logarithmic replots of these data yielded a straight line with a slope of 1.1. This implies that inactivation results from the modification of a single class of sites on the enzyme. The pseudo first-order rate constant for ADP-ribosyltransferase inactivation increased with increasing pH from 6.0 to 7.0 and little change was observed between pH 7.0 and 8.0. An apparent pK_a of the modified groups was calculated as 6.97. Studies with imidazole as a model compound yielded a pK_a of 6.95. This suggests that DEP is reacting with a single class of histidyl groups on the transferase. Consistent with this finding is the observation that inactivated enzyme could be reactivated with 0.2 M hydroxylamine at pH 6.5. The addition of sodium chloride or potassium trichloroacetate salts, known to activate enzyme, resulted in a 73% increase in the k_{obs} for ethoxyformylation. There was, however, a considerable difference in the magnitude of the effect of 300 mM NaCl on transferase activity (> 400% increase) and on DEP inactivation (k_{obs} increased 73%). This is consistent with the conclusion that the structural requirements (conformation, dissociation) for accessibility of the sites through which DEP inactivates the enzyme are different from those that determine substrate accessibility and for catalytic function. It appears that at least a portion of the catalytic site is relatively inaccessible in the inactive associated form of the enzyme and more accessible where the enzyme is in the dissociated active protomeric form, e.g., in the presence of salt or histone.

Purification of a new NAD:Arginine ADP-Ribosyltransferase in Turkey Erythrocyte. Previous results from this laboratory have shown the purification of a soluble NAD:arginine ADP-ribosyltransferase from turkey erythrocytes. This enzyme is activated by the presence of histone or chaotropic salts. Activation is thought to result from the conversion of inactive oligomers of the transferase to active protomer. During the purification of this enzyme, another transferase activity was observed, one which was not affected by salt or histone. This new enzyme was purified by chromatography on phenyl-Sepharose, concanavalin A-Sepharose, carboxymethyl cellulose, Procion Red Agarose, and finally gel filtration on AcA 54. The enzyme exhibited one protein-staining band on SDS

gel electrophoresis of 34,000 daltons. The molecular weight of this enzyme was unaffected by the presence of salt or histone. The product of the reaction catalyzed by the enzyme was positively identified as arginine-ADP-ribose by HPLC on an anion-exchange column.

Significance to Biomedical Research: Hormone-dependent adenylate cyclase systems are responsible for the regulation of many metabolic processes in numerous tissues including heart and lung. Cyclase systems include hormone receptors (R), guanyl nucleotide-binding regulatory subunit (G), and catalytic subunit (C). To fully understand the regulation of adenylate cyclases, the several components must be probed independently. Choleragen activates adenylate cyclase by catalyzing the ADP-ribosylation of G. Presumably, ADP-ribosyl-G activates C directly, bypassing R; therefore, factors that influence the interaction of G with C could be studied independently of R.

Avian erythrocytes contain enzymes which catalyze ADP-ribosyltransferase reactions similar to that of choleragen. Such enzymes may also serve as endogenous regulators of metabolism. A knowledge of factors which affect the activity of ADP-ribosyltransferase may suggest how these enzymes are involved in intracellular metabolic regulation.

Proposed Course: 1) To probe further the interaction between the G and C subunits of adenylate cyclase; 2) to continue the study of eukaryotic ADP-ribosyltransferases with respect to role of effectors, tissue distribution, and subcellular location.

Publications:

Watkins, P.A., Moss, J., and Vaughan, M.: ADP ribosylation of membrane proteins from human fibroblasts: Effect of prior exposure of cells to choleragen. J. Biol. Chem. 256: 4895-4899, 1981.

Nakaya, S., Watkins, P.A., Bitonti, A.J., Hjelmeland, L.M., Moss, J., and Vaughan, M.: GTP stabilization of adenylate cyclase activated and ADP-ribosylated by choleragen. Biochem. Biophys. Res. Commun. 102: 66-74, 1981.

Watkins, P.A. and Moss, J.: Effects of nucleotides on activity of a purified ADP-ribosyltransferase from turkey erythrocytes. Arch. Biochem. Biophys. 216: 74-80, 1982.

Pekala, P.H., Lane, M.D., Watkins, P.A., and Moss, J.: On the mechanism of preadipocyte differentiation. Masking of poly(ADP-ribose) synthetase activity during differentiation of 3T3-L1 preadipocytes. J. Biol. Chem. 256: 4871-4876, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00627-04 CM

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Interaction of GTP-Binding Proteins and Catalytic Component of Adenylate Cyclase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Bruce I. Terman	Staff Fellow	CM	NHLBI
	Joel Moss	Head, Section on Molecular Mechanisms	CM	NHLBI
	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI

COOPERATING UNITS (if any)

Dr. Leonard Hjelmeland, NICHHD, NIH.

LAB/BRANCH
Cellular Metabolism

SECTION
Molecular Mechanisms

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Regulation of the catalytic unit (C) of adenylate cyclase through interaction with both the G/F component and by cytosolic agents is important in understanding the mechanism by which hormones, cholera toxin, calmodulin, other agents stimulate cellular cAMP production. To study that regulation, we are attempting to isolate and characterize C from various tissues. The requirements for stability and activity of C from bovine brain were investigated. C, separated from G/F by gel filtration of a CHAPS-solubilized particulate fraction, is inactivated by incubation at 30°C; ATP provided almost complete stabilization. Whereas catalytic activity in these preparations is virtually completely dependent on the presence of Mg-2+ or Mn-2+, stabilization by ATP does not require divalent cations. Calcium plus calmodulin and phosphatidylcholine plus lysophosphatidylcholine also stabilize C. In addition, these agents activate C. These findings, in addition to defining some properties of C that are apparently independent of G/F, may well find practical application in procedures for purification and/or further characterization of C.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: While it has become clear that the direct effect of many hormones on cells is the stimulation of the enzyme adenylate cyclase, the molecular mechanism leading from hormone binding to cAMP production has yet to be clarified. As a prerequisite to clarifying this mechanism, we are attempting to purify the catalytic unit of adenylate cyclase from animal tissues and to clarify the cellular regulation of the enzyme.

Methods Employed: C and G/F are solubilized from bovine brain cortex with 13 mM CHAPS followed by centrifugation at 100,000 x g for 60 min. C and G/F are readily separated from each other by gel filtration of the solubilized particulate fraction on Ultrogel AcA 34 in the presence of CHAPS. Fractions containing C, which emerge in the void volume, are pooled and used immediately for experiments. Adenylate cyclase activity in the pooled fraction is not stimulated by Gpp(NH)p and does not reconstitute Gpp(NH)p-stimulated cyclase activity when combined with AC$^-$ membranes. By these criteria it is concluded that the pooled fraction from the gel filtration column lacks functional G/F.

The stability of C from bovine brain membranes was studied by incubation of C at 0°C or 30°C in the presence or absence of potential stabilizing agents.

Adenylate cyclase activity was assayed by the procedure of Salomon et al. (Anal. Biochem. 58: 541, 1974).

Major Findings: CHAPS-solubilized bovine brain C, separated from G/F by gel filtration chromatography, is inactivated by incubation at 30°C; 0.5 mM ATP provided almost complete stabilization. Whereas catalytic activity in these preparations was virtually completely dependent on the presence of Mg^{2+} or Mn^{2+}, stabilization by ATP did not require divalent cations.

Activity of the Ultrogel AcA 34-purified C was increased by calmodulin plus calcium and phosphatidylcholine plus lysophosphatidylcholine. It is concluded that the locus of action of these stimulatory agents upon adenylate cyclase is at C, not at G/F. The concentration of phospholipids which gave maximal cyclase activity was determined, and it was found that phospholipids are required for maximal calmodulin plus calcium stimulation.

Calcium plus calmodulin and phosphatidylcholine plus lysophosphatidylcholine partially prevented inactivation of C at 30°C. The protection afforded by calmodulin plus phospholipids or calmodulin plus ATP was significantly greater than that provided by either alone.

These findings, in addition to defining some properties of C that are apparently independent of G/F, may well find practical application in procedures for purification and/or further characterization of C.

Significance to Biomedical Research: Many hormones, drugs, and other agents exert their effects on cells by modifying cyclase activity. Our work will help clarify the biochemical mechanism by which that modification takes place in both normal and diseased states where the modification is altered.

Proposed Course: To further clarify the biochemical mechanism by which hormones and other agents regulate adenylate cyclase activity.

Publication: Bitonti, A.J., Moss, J., Hjelmeland, L., and Vaughan, M.: Resolution and activity of adenylate cyclase components in a zwitterionic cholate derivative [3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate]. Biochemistry, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00630-03 CM

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Metabolism of Lipids in Human Fibroblasts Grown in Culture

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Joel Avigan	Research Chemist	CM	NHLBI

COOPERATING UNITS (if any)

Neonatal and Pediatric Medicine Branch, NICHHD

LAB/BRANCH

Cellular Metabolism

SECTION

Metabolic Regulation

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.3	0.2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

An increased content of very long chain fatty acids (VLCFA), namely of lignoceric and cerotic acids, was confirmed in a number of fibrolast cell lines derived from patients with adrenoleukodystrophy (ALD). ALD cells oxidized labeled palmitate, lignocerate, or cerotate at a lower rate than normal cells, but the reduction in the metabolism of VLCFA was only moderately larger (by 40-60%) than the reduction in palmitate oxidation. The oxidation of VLCFA was shown to depend on carnitine, which demonstrates mitochondrial involvement in this process.

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PHS-6040
(Rev. 2-81)

Project Description:

Objectives and Background: The objective of this project is the study of abnormalities in lipid metabolism in certain diseases which are often manifested by accumulation of lipids in muscle (myopathic carnitine deficiency) or increased content of certain unusual fatty acids normally present in trace amounts only (very long chain fatty acids in adrenoleukodystrophy (ALD) and adrenomyeloneuropathy (AMN)).

Methods Employed: Human skin fibroblasts derived from either normal or affected individuals were grown by standard methods. Oxidation of labeled caprylic, palmitic, lignoceric, or cerotic acids was assayed in cell monolayers by a method previously developed in our laboratory. Mixed leukocytes were prepared from patients' blood by a conventional method.

Major Findings: Measurement of total very long chain fatty acid (VLCFA) content of four male control and three ALD fibroblast cell lines was carried out by gas liquid chromatography on a capillary column. To correct for differences in analytical recoveries, results were expressed as ratios of C-26:C-22 and C-24:C-22 (the content of behenic acid (C-22) is not affected by the disease). The C-26:C-22 ratio was 0.066 ± 0.038 (S.D.) for normal cells and 0.43 ± 0.13 for ALD cells (a 6- to 7-fold increase) while the C-24:C-22 ratios were 2.1 ± 0.5 and 3.3 ± 0.5, respectively. These findings were consistent with those of Kawamura et al. (Biochem. Biophys. Res. Commun. 82, 114, 1978).

To determine whether the increased concentration of VLCFA was due to a catabolic defect, the rate of oxidation of 1-^{14}C-lignoceric acid (C-24) and 1-^{14}C-cerotic acid (C-26) was assayed in normal and ALD fibroblasts. Cells from five ALD patients oxidized all the labeled fatty acids at a slower rate than cells from normal individuals that were run in parallel. The ratio of ALD:normal was for palmitate 0.73 ± 0.47 (S.D.), for lignocerate 0.27 ± 0.13, for cerotate 0.42 ± 0.06. The above results indicate a relatively small specific effect of the disease on metabolism of VLCFA compared with that of palmitate. In a single experiment with ALD- and normal-mixed leukocytes, no difference in cerotate metabolism was observed. No defect in oxidative metabolism was found in fibroblasts of an AMN patient. Because of speculation in literature that oxidation of VLCFA is extramitochondrial and is, therefore, subject to a specific metabolic defect which does not affect metabolism of the prevalent long chain fatty acids, we tested the dependence of the metabolism of cerotate on carnitine. Using carnitine-depleted fibroblasts (see Z01 HL 00630-02 CM), it was found that addition of carnitine stimulated to a similar degree the oxidation of palmitate and cerotate in either normal or ALD cells and that the metabolism of lignocerate was also affected by carnitine. These effects suggested that mitocondria are the site of metabolism of VLCFA and that ALD cells are normally responsive to carnitine.

It was shown that oxidation of caprylic acid (8 carbon chain) in fibroblast is not dependent on carnitine. This would suggest that lipids containing caprylic acid could be a preferred source of energy in patients with muscle carnitine deficiency. The empirically found beneficial effect of dietary medium chain triglycerides in patients with muscle carnitine deficiency may be

due to such independence of caprylic acid as metabolic substrate with respect to carnitine.

Significance to Biomedical Research: Elucidation of biochemical basis for the diseases linked with abnormalities in lipid metabolism is likely to contribute to solutions of related medical problems.

Proposed Course: Additional studies are intended, aiming to determine the precursor of the very long chain fatty acids in ALD and AMN.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00632-02 CM

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Regulation of Hydroxymethyl CoA Reductase in Human Skin Fibroblasts

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Joel Avigan	Research Chemist	CM	NHLBI
	Zafarul H. Beg	Research Chemist	MD	NHLBI

COOPERATING UNITS (if any)

Molecular Disease Branch, NHLBI

LAB/BRANCH

Cellular Metabolism

SECTION

Metabolic Regulation

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	0.7	0.6

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Various inhibitors (sterols, bile acids) of cholesterol synthesis in cultured human skin fibroblasts cause within 10-30 minutes phosphorylation of HMGCoA reductase and a concomitant depression of its expressed activity with the total activity remaining unchanged. Longer periods of incubation of fibroblasts with these inhibitors reduce progressively the total activity of the enzyme. Active HMGCoA reductase kinase is required for the phosphorylating action and the kinase is active only in its phosphorylated form.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: The previously reported phase of this project (Z01 HL 00632-01 CM) was extended to include experiments in which a variety of factors was studied for their effects on phosphorylation (reversible inactivation) of HMGCoA reductase in human fibroblasts grown in culture and for the role of this process in the biological regulation of this enzyme.

Methods Employed: The methods were similar to those described previously. Cells grown as monolayers were incubated for 20-24 hr with MEM containing 10% solvent-extracted serum. In a typical experiment, some dishes were incubated with an inhibitor, others were kept as separate controls for each incubation time. Cells were washed and harvested in the presence of 0.1 M KF to inhibit the action of endogenous phosphatase and were assayed for HMGCoA reductase activity. Preincubation with phosphoprotein phosphatase purified from rat liver cytosol was carried out in some samples when the total activity of dephosphorylated enzyme was measured.

Major Findings: It was demonstrated that in cultured human fibroblasts all the inhibitors of cholesterol synthesis tested so far caused an initial phosphorylation of the enzyme which depressed its expressed activity but not its restorable total activity. When the inhibitor was allowed to act for a longer time, however, the total activity became also progressively depressed. Of the inhibitors tested, the most potent one was 25-hydroxycholesterol, which added at a concentration as low as 0.4 µg/ml inhibited significantly within 10 min the expressed activity of HMGCoA reductase. Seven-ketocholesterol, a known inhibitor of cholesterol synthesis, was similarly active. Incubation of fibroblasts with 2.5 mM glycocholic acid diminished rapidly enzyme activity. Complete reversal of inhibition was seen only in cell cultures incubated with the inhibitor for 10 min; in cells incubated longer, the total activity of the enzyme became progressively depressed. In other experiments, it was demonstrated that active HMGCoA reductase-kinase phosphorylated the reductase in either normal or FH homozygous fibroblasts. Dephosphorylation through the action of phosphatase of the reductase-kinase caused inactivation of the kinase.

Significance to Biomedical Research: The mechanism regulating the activity of enzymes controlling cholesterol biosynthesis is relevant to medical problems associated with abnormalities in steroid metabolism.

Proposed Course: Studies are in progress to quantify the effect of specific antibodies against HMGCoA reductase on its activity in cells undergoing inhibition by LDL, aiming to detect the presence (or absence) of an immunochemically reactive but enzymatically inactive pool of enzyme.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00633-02 CM

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

The Mechanism of Action of Choleragen

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Drusilla L. Burns	Staff Fellow	CM	NHLBI
OTHERS:	Joel Moss	Head, Section on Molecular Mechanisms	CM	NHLBI
	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Cellular Metabolism

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Choleragen activates adenylate cyclase by catalyzing the ADP-ribosylation of a guanyl nucleotide-binding regulatory protein (G/F). Choleragen and certain hormones are believed to activate adenylate cyclase, at least in part, by decreasing the affinity of G/F for guanyl nucleotides. Consistent with this hypothesis were the observations that both choleragen and certain hormones stimulate the release of a pool of GDP from turkey erythrocyte membranes. It had not been demonstrated that this pool of GDP was indeed released from G/F. We have now obtained evidence that the protein which releases GDP upon incubation with choleragen is identical to G/F. After solubilization, the protein which releases GDP upon choleragen treatment cochromatographed with G/F activity. Moreover, the only protein which chromatographed as a protein of this size and which was ADP-ribosylated by choleragen was G/F. These data provide further evidence that choleragen treatment decreases the affinity of G/F for GDP. The kinetics of toxin-stimulated release of GDP from membranes was also examined.

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PHS-6040
(Rev. 2-81)

Project Description:

Objectives: Adenylate cyclase plays a critical role in the control of cellular metabolic processes. Turkey erythrocyte adenylate cyclase is activated by either β-adrenergic agonists or choleragen (cholera toxin). Both are thought to act through the guanyl nucleotide-binding regulatory protein (G/F) of the adenylate cyclase complex; β-adrenergic agonists such as isoproterenol bind to receptors which in turn are believed to interact with G/F while choleragen catalyzes the ADP-ribosylation of a 42,000 dalton subunit of G/F. The alterations in G/F caused by receptor binding and ADP-ribosylation are not well-understood. It is currently believed that the catalytic unit of adenylate cyclase is active when GTP is bound to G/F. Hydrolysis of the bound GTP to GDP by a specific GTPase terminates this activation; reactivation is dependent on dissociation of GDP to permit binding of GTP. Thus, a decrease in the affinity of G/F for GDP would promote the binding of GTP, resulting in increased adenylate cyclase activity. Consistent with that hypothesis were the observations that treatment of turkey erythrocyte membranes with either choleragen (in the presence of NAD) or isoproterenol resulted in an increase in the rate of release of GDP from sites on those membranes (Cassel, D. and Selinger, Z.: Proc. Natl. Acad. Sci. USA 75: 4155-4159, 1978). It has not been demonstrated, however, that this pool of GDP was indeed released from G/F. In the studies reported here, we have investigated the identity of the protein which releases guanyl nucleotides upon incubation with isoproterenol or choleragen as well as the kinetics of this release.

Methods Employed: Binding of radiolabeled guanyl nucleotides and assay of release of labeled nucleotides was conducted as described previously.

G/F activity was measured by its ability to stimulate the activity of adenylate cyclase catalytic units of cyc^- cell membranes in the presence of guanyl nucleotides (Ross, E., Maguire, M., Sturgill, T., Biltonen, R., and Gilman, A.: J. Biol. Chem. 252: 5761-5775, 1977). G/F was also detected by incubating membranes with choleragen and [^{32}P]NAD in order to [^{32}P]ADP-ribosylate a 42,000 dalton subunit of G/F. Labeled membrane proteins were separated by SDS gel electrophoresis. The radiolabeled subunit of G/F was visualized by autoradiography or quantified by slicing the gels and determining the amount of radioactivity migrating as a protein having a molecular weight of 42,000.

Adenylate cyclase activity was measured according to the method of Salomon et al. (Salomon, G., Londos, C., and Rodbell, M.: Anal. Biochem. 58: 541-548, 1974).

Major Findings: Both choleragen and β-adrenergic agonists, two agents which activate turkey erythrocyte adenylate cyclase, have been reported to accelerate release of bound [^{3}H]guanyl nucleotides from turkey erythrocyte membranes. We have now obtained evidence that choleragen- or isoproterenol-stimulated release reflects a change in the affinity of the regulatory subunit (G/F) of adenylate cyclase for guanyl nucleotides. Preparations of turkey erythrocytes that had bound radiolabeled GTP were solubilized with 1% Lubrol PX and chromatographed on Ultrogel AcA 34. The protein from which guanyl nucleotide was released upon incubation with choleragen or isoproterenol was coeluted with adenylate cyclase activity measured under conditions which would reflect the

presence of G/F. Furthermore, this protein appears to be the same size as the complex containing the 42,000 dalton subunit of G/F, ADP-ribosylated by choleragen, and is presumably a subunit of G/F. G/F was the only protein which both chromatographed as a protein of this size and which was ADP-ribosylated by the toxin.

ADP-ribosylation of the 42,000 dalton subunit of G/F by choleragen occurred with a half-time of about 5 min, whereas choleragen-stimulated release of guanyl nucleotides was much slower ($t_{1/2}$ 60 min). When membranes were treated with choleragen and NAD, the delay in activation of adenylate cyclase by guanylyl imidodiphosphate was decreased but not abolished, a finding consistent with the idea that release of endogenously bound nucleotide (and subsequent binding of the nonhydrolyzable GTP analog) occurs only slowly following ADP-ribosylation. In contrast, activation of the adenylate cyclase of either toxin-treated or untreated membranes in the presence of isoproterenol and guanylyl imidodiphosphate was very rapid. These data support the hypothesis that isoproterenol and choleragen may activate adenylate cyclase, at least in part, by increasing the rate of release of guanyl nucleotides from G/F.

Significance to Biomedical Research: Adenylate cyclase plays a critical role in many tissues including the heart and lung by mediating the actions of certain hormones. In addition, certain bacterial toxins, including choleragen, exert their effects on cells by altering the activity of this enzyme. This study aids in the understanding of the molecular mechanisms by which choleragen and these hormones activate adenylate cyclase.

Proposed Course: We hope to investigate further the hormonal regulation of adenylate cyclase.

Publications:

Burns, D.L., Moss, J., and Vaughan, M.: Choleragen-stimulated release of guanyl nucleotides from turkey erythrocyte membranes. J. Biol. Chem. 257: 32-34, 1982.

Moss, J., Burns, D.L., Chang, P.P., Cutilletta, A.F., and Vaughan, M.: Characterization of the GTP-binding component of the adenylate cycalse system in isolated myocardial muscle cells. In Proceedings of the Vth US:USSR Symposium "Cellular Biology of Heart," Problem Area 3, "Myocardial Metabolism," June 8-10, 1981, Hershey Pa., in press.

Moss, J., Burns, D.L., and Vaughan, M.: Mechanism of action of choleragen: Effect of toxin on binding of guanyl nucleotides. In Proceedings of the International Symposium on Bacterial Diarrheal Diseases, Osaka, Japan, March 23-25, 1982, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00634-02 CM

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Purification and Characterization of cGMP-Stimulated Cyclic Nucleotide Phosphodiesterase from Calf Liver

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Toshihiko Yamamoto	Visiting Fellow	CM	NHLBI
	Vincent C. Manganiello	Head, Section on Biochemical Physiology	CM	NHLBI
	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Cellular Metabolism

SECTION
Biochemical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.4	1.0	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A cGMP-stimulated cyclic nucleotide phosphodiesterase has been purified more than 13,000-fold to apparent homogeneity from calf liver. The purified enzyme exhibited a single protein-staining band in SDS-polyacrylamide gel electrophoresis with a molecular weight of 102,000, a sedimentation coefficient of 6.9S, and Stokes' radius of 67 A. From these values an apparent molecular weight of 201,000 and a frictional ratio (f/fo) of 1.7 were calculated. These hydrodynamic properties suggest that the native enzyme exists as a nonspherical dimer of similar, if not identical, polypeptide chains. The apparent Km's were 22 and 40 μM for cGMP and cAMP, respectively. The Vmax's and turnover numbers were 87 μmol/min/mg of protein, 17,500/min for cGMP and 79 μmol/min/mg of protein, 15,800/min for cAMP, respectively. The hydrolysis of both cyclic nucleotides exhibited positive homotropic cooperativity with Hill coefficients of 1.8 for cAMP and 1.2 for cGMP. The rate of cAMP hydrolysis by the purified phosphodiesterase when measured at 0.5 μM cAMP was enhanced more than 30-fold by cGMP with an apparent activation constant (Ka) of 0.35 μM.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: To purify the cGMP-stimulated cyclic nucleotide phosphodiesterase (EC 3.4.1.17) and characterize the physical and enzymatic properties of the protein.

Methods Employed: The Purification Procedure. Chromatography on DEAE-cellulose, precipitation with ammonium sulfate, and chromatography on columns of DEAE-Sepharose CL-6B, C^8-$H_2N(CH_2)_2NH$-cAMP-agarose and N^6-$H_2N(CH_2)_2$-cAMP-agarose were utilized.

Estimation of Purity and Identification of the Enzyme. The purity of the enzyme was examined by analytical polyacrylamide disc gel electrophoresis, sucrose density gradient centrifugation, and chromatography on Sepharose CL-6B and polyacrylamide slab gel electrophoresis in SDS.

Physicochemical Properties of the Enzyme. The sedimentation constant and the Stokes' radius were estimated by sucrose density gradient centrifugation and chromatography on Sepharose CL-6B, respectively.

Major Findings: The cGMP-stimulated cyclic nucleotide phosphodiesterase could be purified more than 13,000-fold from calf liver extracts with a final yield of 5%. The purified enzyme exhibited a single protein-staining band in SDS-polyacrylamide gel electrophoresis with an M_r of 102,000.

Phosphodiesterase activity comigrated with the protein-staining band on analytical polyacrylamide gel electrophoresis, sucrose density gradient centrifugation and gel filtration.

The purified phosphodiesterase exhibited a sedimentation coefficient of 6.9S and Stokes' radius of 67Å from which values an M_r of 201,000 and a frictional ratio (f/fo) of 1.7 have been calculated (assuming a partial specific volume of 0.74 mg/ml). These hydrodynamic properties suggest that the native enzyme exists as a nonspherical dimer of similar, if not identical, polypeptide chains.

The purified enzyme lacked absolute substrate specificity, hydrolyzing both cGMP and cAMP to a comparable degree. The concentrations of cGMP and cAMP at half-maximal velocities were 22 and 40 μM, respectively. The maximal velocities were 87 μmol/min/mg for cGMP and 79 μmol/min/mg for cAMP, respectively.

Calculated turnover numbers were 1.75×10^4 min^{-1} (cGMP) and 1.58×10^4 min^{-1} (cAMP) assuming a molecular weight of 201,000 daltons.

Apparent Hill coefficients (n_{app}) were calculated at 1.8 for cAMP and 1.2 for cGMP, suggesting positive homotropic cooperativity for hydrolysis of both cyclic nucleotides.

At a substrate concentration of 0.5 μM [^{3}H]cAMP, a wide range of cGMP concentrations (.010-500 μM) stimulated cAMP hydrolysis. Stimulation approached 32-fold at 4 M cGMP and the apparent activation constant (K_a) of cGMP for cAMP hydrolysis was 0.35 μM.

Significance to Biomedical Research: Many biochemical effectors, such as hormones, are known to exert their effects on target cells by altering metabolism of cyclic nucleotides. The present study of the phosphodiesterase involved in the degradation of the cyclic nucleotides is important for understanding the mechanism that regulates physiological and pathological processes in mammalian cells.

Proposed Course: Continued characterization of the enzyme including study of allosteric regulatory mechanism.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00635-01 CM

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Interaction of Muscarinic Receptor and GTP-Binding Proteins of Adenylate Cyclase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Judith Hsia	Medical Staff Fellow	CM	NHLBI
OTHERS:	Joel Moss	Head, Section on Molecular Mechanisms	CM	NHLBI
	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Cellular Metabolism

SECTION
Metabolic Regulation

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The actions of hormones and drugs which inhibit adenylate cyclase are mediated by specific receptors and an inhibitory GTP-binding regulatory protein (GI). Guanine nucleotides decrease the affinity of receptors for their ligands in many systems. This is thought to indicate that the receptor is coupled to GI. Although other labs have demonstrated this phenomenon for the muscarinic receptor in membranes, we have solubilized the receptor-GI complex from rat heart membranes and have demonstrated guanine nucleotide sensitivity of receptor affinity in the solubilized system. Further, we have separated the receptor-GI complex from uncoupled receptor and are continuing to characterize these proteins in an attempt to elucidate the molecular mechanism of adenylate cyclase inhibition.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: When many hormones bind to their target cells, the ligands' presence is signalled across the plasma membrane by adenylate cyclase. Stimulation of adenylate cyclase is mediated by specific receptors and a stimulatory guanine nucleotide regulatory protein (GS). It has been shown in the β-adrenergic system that this stimulatory receptor binds β-agonists with lower affinity in the presence of guanine nucleotides, indicating coupling of the receptor to GS. It is possible that an analogous system exists for adenylate cyclase inhibition in that specific receptors for inhibitory hormones couple to an inhibitory guanine nucleotide-binding protein, GI.

Coupling of muscarinic receptor, which mediates adenylate cyclase inhibition, to GI has been investigated by others in membranes from a variety of tissues but has not previously been studied in a solubilized system. Solubilization is valuable in that it allows separation of the receptor-GI complex from receptor alone. By separating and studying these proteins, we hope to understand better the mechanism by which adenylate cyclase is inhibited.

Methods Employed: Solubilization of the receptor-GI complex required a preliminary incubation step followed by agonist-promoted coupling. After solubilization in digitonin and cholate, the material was concentrated and chromatographed on AcA 34. Solubilized receptor was assayed by specific ^{3}H-scopolamine binding. Bound ^{3}H-scopolamine was separated from unbound by desalting over Sephadex.

Major Findings: Heart membranes contain significant amounts of endogenous GTP. The agonist-promoted coupling of muscarinic receptor to G_I was found to be augmented by prior incubation of membranes to release endogenous GTP. Coupling is then achieved by treating the membranes with oxotremorine, a potent muscarinic agonist. Coupling does not occur if GppNHp, a nonhydrolyzable GTP analogue, is present along with agonist. The coupled complex is solubilized in digitonin and cholate and chromatographed on AcA 34 Ultrogel.

The elution profile of specific ^{3}H-scopolamine binding has three major peaks. Fractions from each peak were pooled and studied for guanine nucleotide effect on receptor affinity. Muscarinic agonist binding of protein from the lowest molecular weight peak was unaffected by guanine nucleotides, suggesting that this protein represents receptor without GT. Proteins from both of the higher molecular weight peaks bind oxotremorine with lower affinity in the presence of GppNHp. Thus, these peaks may represent forms of a coupled receptor-GI complex. Why there are two such species remains to be determined.

Significance to Biomedical Research: Adenylate cyclase is a ubiquitous enzyme that plays a critical role in mediating the actions of a variety of hormones. Our study has provided new information to aid in elucidation of the mechanism whereby hormones inhibit adenylate cyclase.

Proposed Course: We plan to continue our study of the mechanism of hormone-mediated adenylate cyclase inhibition.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00636-01 CM

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Regulation of Particulate cAMP Phosphodiesterase in 3T3-L1 Fatty Fibroblasts

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Martha Elks	Clinical Assocate	CM	NHLBI
	Vincent C. Manganiello	Head, Section on Biochemical Physiology	CM	NHLBI
	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Cellular Metabolism

SECTION
Biochemical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

3T3-L1 cells were treated with methylisobutylxanthine, dexamethasone and insulin to induce fatty metamorphosis. Incubation of differentiated 3T3-L1 cells with 100 pM insulin, 10 μM epinephrine, 0.1 mM adenosine, 15 ng/ml TSH, or 2 μU/ml ACTH rapidly increases the activity of a particulate cAMP phosphodiesterase. Incubation of differentiated cells with dexamethasone (10 nM for 72 hr) blocks the effect of insulin but not of epinephrine; higher concentrations of dexamethasone (at least 100 nM for 72 hr) are required to inhibit the effect of epinephrine.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: To investigate the mechanism of activation of particulate cAMP phosphodiesterase in 3T3-L1 fatty fibroblasts by insulin and other hormones.

Methods Employed: 3T3-L1 fatty fibroblasts, obtained from Dr. M. D. Lane of Johns Hopkins University, were grown to confluence in six-well culture plates. Two days later, the cells were treated with 1 μM dexamethasone, 1 μM insulin, and 0.1 mM methylisobutylxanthine for 72 hr. These agents were removed and incubation continued with fresh growth medium. Within a week the cells developed large fat deposits and enzyme activities characteristic of mature adipocytes. At various stages in the postconfluent development of the adipocyte phenotype, cells were exposed to various hormones for defined periods of time, harvested by scraping with rubber policeman, homogenized in Tris/sucrose/magnesium/EGTA buffer and centrifuged (100,000 x g for 45 min). Samples of supernatant or particulate fractions were assayed for cAMP or cGMP phosphodiesterase activities.

For investigation of the activation of the phosphodiesterase by hormones in subcellular fractions, the cells were harvested and homogenized in a Tris/magnesium/sucrose buffer, pH 7.8, and then centrifuged (100,000 x g for 45 min). The pellet fraction was suspended in Tris/magnesium/sucrose/calcium buffer. Hormones were added directly to this fraction under various conditions. After incubation for appropriate periods of time, samples were taken for assay of phosphodiesterase activities.

Major Findings: Incubation of 3T3-L1 fatty fibroblasts for 8 min with 100 pM insulin, 1 μM epinephrine, ACTH (2 μU/ml), or TSH (15 ng/ml) increased by 30-70% the activity of a particulate phosphodiesterase (assayed with 0.5 μM [^{3}H]cAMP). Activation of the particulate phosphodiesterase by hormones was first observed about 7 days after initiation of differentiation by treatment (72 hr) with isomethylbutylxanthine, dexamethasone, and insulin and was maximal at 10-14 days after initiation of treatment. During initiation of differentiation, soluble cGMP PDE activity was reduced by 50% but was restored to predifferentiation values over the next 7-10 days. In differentiated cells, incubation with insulin or epinephrine did not alter either soluble cAMP PDE activity or particulate or soluble cGMP PDE activities. In confluent, nondifferentiated 3T3-L1 fibroblasts, incubation with insulin or epinephrine did not alter particulate cAMP PDE activity.

The increase in particulate PDE activity was maximal after incubation of cells with insulin or epinephrine for 6-8 min. The increase in phosphodiesterase activity persisted for at least 30 min in the presence of insulin. The increase in phosphodiesterase activity produced by epinephrine persisted for about 10-15 min and then declined despite the continued presence of the hormone.

Maximal activation was produced by incubation of cells with 0.1 to 1 nM insulin. Higher concentrations produced smaller increments in PDE activity and with 100 nM insulin PDE activity was not increased at all. Half-maximal effects of epinephrine were produced with ~ 0.1 μM epinephrine and maximal effects with 1 μM. Propranolol inhibited the action of epinephrine. Dose-response relationships for effects of ACTH, TSH, and adenosine have been established.

Incubation of fatty fibroblasts with dexamethasone 0.01 to 1 μM for 48-72 hr had little or no effect on basal particualte PDE activity. Incubation with 10^{-6} dexamethasone for 48 hr inhibited activation of the particulate PDE by 10 μM epinephrine or 100 pM insulin. Treatment with dexamethasone reduced the sensitivity to insulin (the dose-response curve for activation was shifted to the right) as well as the maximal increase in activity produced by insulin. Incubation with lower concentrations of dexamethasone 0.01 or 0.1 μM for 72 hr inhibited activation by insulin and 0.1 μM (but not 0.01 μM) dexamethasone inhibited the effect of epinephrine.

Significance to Biomedical Research: Cellular cAMP is an important messenger in the control of multiple metabolic processes in virtually all mammalian cells. Local availability of cAMP is dependent upon the rate of its production by adenylate cycalse and its degradation by phosphodiesterases. Thus, control of activity of these enzymes is central to the control of metabolic balance in a cell. Also, investigation of the mechanism by which hormones control subcellular enzyme activity is central in the understanding of endocrine function.

Proposed Course: Studies are continuing to eludicate the mechanism of this effect of dexamethasone and its consequences for cell cAMP content. Further attempts will be made to establish a system in which direct effects of insulin on the particulate PDE can be demonstrated with the goal of defining the mechanism of this effect.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00637-01 CM

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Calmodulin-Dependent Phosphodiesterase: Mechanism of Activation by Proteolysis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Irene E. Stith-Coleman	PRAT Fellow	CM	NHLBI
	Randall L. Kincaid	Staff Fellow	CM	NHLBI
	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI

COOPERATING UNITS (if any)
Dr. Stith-Coleman was in the Pharmacology/Toxicology Program, NIGMS, NIH, until July 1, 1982.

LAB/BRANCH
Cellular Metabolism

SECTION
Metabolic Regulation

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Purified phosphodiesterase from bovine and ovine brain exhibited a single peptide of about 59,000 daltons on sodium dodecyl sulfate gel electrophoresis. Limited proteolysis with chymotrypsin produced peptides of 57,000 and 45,000 daltons (bovine) and 55,000, 53,000 and 38,000 daltons (ovine). The final stable peptides (45K bovine, 38K ovine) were fully active without calmodulin and calcium. Substrate, cGMP, or cAMP, with or without magnesium, had no effect on the pattern of proteolysis. Glycerol (20%) markedly reduced the rate of proteolysis but did not alter the pattern. In the presence of calmodulin plus calcium, however, the final stable peptides were 47,000 daltons (bovine) and 43,000 daltons (ovine). These results suggest differences in the structure of phosphodiesterases of bovine and ovine brain. They further provide direct demonstration of a conformational change in phosphodiesterase in the presence of calmodulin plus calcium which alters the peptide bonds that are accessible to chymotryptic cleavage. Effects of the phosphodiesterase inhibitor trifluoperazine on proteolytic cleavage have also been investigated.

Project Description:

Objectives: To use limited proteolysis as a tool to understand the mechanism of interaction of phosphodiesterase and calmouldin plus calcium.

To characterize the mechanism of inhibition and activation of calmodulin-sensitive phosphodiesterase by trifluoperazine.

Methods Employed: Phosphodiesterase was purified and assayed by published methods developed in this laboratory. Calmodulin was purified from bovine brain by an unpublished method developed in this laboratory. Sodium dodecyl sulfate gel electrophoresis was carried out on trichloroacetic acid-precipitated samples by standard procedures.

Major Findings: Previous studies in this laboratory showed that incubation of calmodulin-sensitive phosphodiesterase, purified from bovine brain, with α-chymotrypsin increased the activity of phosphodiesterase to the level observed with calmodulin plus calcium and generated a stable peptide of ~ 45,000 daltons that did not bind to calmodulin-Sepharose. These studies were extended to compare the effects of limited proteolysis by α-chymotrypsin on bovine and ovine brain phosphodiesterase. Preparations of phosphodiesterase from bovine and ovine brain exhibited a single peptide of ~ 59,000 daltons on SDS gel electrophoresis. However, the final stable peptides and intermediate products of chymotryptic proteolysis were different (bovine, 59,000 → 57,000 → 45,000; ovine, 59,000 → 55,000 → 53,000 → 38,000). These findings indicate differences in the primary structure of bovine and ovine enzymes. Both final peptides (bovine 45K, ovine 38K) appeared to be quite stable, since incubation with α-chymotrypsin up to 60 min produced no new peptides and the enzymes remained fully active. Glycerol (up to 20%) significantly reduced the rate of proteolysis but did not alter the pattern of fragmentation. Proteolysis of phosphodiesterase in the presence of calmodulin plus calcium produced final stable peptides of 47,000 daltons from the bovine and 43,000 daltons from the ovine enzyme. These peptides were never observed in the absence of calmodulin plus calcium. The presence of substrate (cGMP or cAMP, with or without Mg^{2+}) had no detectable effect, quantitative or qualitative, on the chymotrypic proteolysis of the phosphodiesterases.

It is generally believed that trifluoperazine (TFP) inhibits calmodulin-sensitive phosphodiesterase activity by competitively binding calmodulin, which is required for activation. In preliminary studies with purified phosphodiesterase, as reported with less pure phosphodiesterase, TFP at concentrations ranging from 10 to 75 μM inhibited calmodulin-stimulated activity. However, at slightly higher concentrations (100 to 200 μM), phosphodiesterase activity was increased to a level comparable to that observed in the presence of calmodulin plus calcium. This activated state was observed both in the presence and absence of calmodulin plus calcium. At concentrations of TFP that maximally inhibit calmodulin-stimulated activity, chymotrypsin treatment (in the presence of calmodulin plus calcium) resulted in the same proteolytic pattern that was observed in the presence of calmodulin plus calcium alone. These results indicate that (1) TFP can activate as well as inhibit calmodulin-sensitive phosphodiesterase, (2) TFP can directly interact with phosphodiesterase in the absence of calmodulin and calcium, and (3) TFP inhibition of calmodulin activation may not be due to interference with the calmodulin-phosphodiesterase interaction.

Significance to Biomedical Research: Cyclic nucleotides are involved in many cellular processes. Phosphodiesterases are responsible for the degradation of these nucleotides and therefore contribute significantly to the maintenance of cyclic nucleotide levels in cells. Certain antipsychotic drugs selectively inhibit the calmodulin-sensitive phosphodiesterase. Knowledge of the mechanism of interaction between phosphodiesterase and calmodulin may contribute to a better understanding of the method by which these drugs alter the activity of this enzyme.

Proposed Course: Kinetic characteristics of peptides produced from phosphodiesterase by α-chymotrypsin in the presence and absence of calmodulin plus calcium will be compared in an attempt to clarify relationships between calmodulin interaction and activation.

Additional studies will be designed to establish the physical mechanisms of inhibition and activation of the enzyme by TFP.

Publications: None

Annual Report of
Laboratory of Chemical Pharmacology
National Heart, Lung, and Blood Institute
October 1, 1981 to September 30, 1982

A long term objective of this Laboratory has been to develop approaches for discovering drugs and other foreign compounds that cause tissue lesions through the formation of chemically unstable metabolites. Nearly a decade ago, we pointed out that treatments that alter the area under the concentration curve of toxic metabolites within target cells should result in parallel changes in the reaction of unstable toxic metabolites with intracellular protein and in the incidence and severity of the toxicity even when the toxicity is caused by the combination of the metabolite with other intracellular components. With this approach, the Laboratory has discovered that tissue necrosis may be caused by unstable metabolites of many different drugs and other foreign compounds. During recent years, the Laboratory has focused its attention on discovering the identity of the toxic metabolites, the enzymes that catalyze their formation and inactivation and the factors that govern the relative severity of the toxicity in different target organs of different species, and strains of animals.

Formation and Decomposition of Unstable Metabolites

Metabolism and toxicity of haloforms - We have previously reported evidence that chloroform ($CHCl_3$) causes hepatic necrosis through the formation of phosgene ($COCl_2$) by way of hydroxytrichloromethane. Prior treatment of rats with methyl butyl ketone or phenobarbital enhances the toxicity of $CHCl_3$ by inducing the synthesis of a cytochrome P-450 having a molecular weight of 48,000 Daltons. We have purified the forms of cytochrome P-450 induced by the ketone and phenobarbital and have found that treatment of them with V-8 protease or chymotrypsin results in virtually identical polypeptide maps. Moreover, both purified forms convert $CHCl_3$ to $COCl_2$ and metabolize warfarin to its various hydroxylated metabolites at the same relative rates. Prior treatment of rats with acetone, which enhances the toxicity of $CHCl_3$, also induces the synthesis of a cytochrome P-450 but whether this cytochrome P-450 is the same as that induced by phenobarbital and methyl butyl ketone remains to be determined.

Recent evidence suggests that $CHCl_3$ causes kidney damage through the formation of a chemically reactive metabolite that combines with glutathione. The severity of the renal toxicity in rats, DBA male mice and C57 male mice parallels the magnitude of the decease in renal GSH. Moreover, $CHCl_3$ is more potent than $CDCl_3$ as a renal toxicant and causes a greater decrease in renal GSH. But kidney preparations *in vitro* are apparently unable to convert chloroform to phosgene.

Although $CHBr_3$ is as toxic as $CHCl_3$ in causing hepatic necrosis, it does not deplete liver of GSH as rapidly as $CHCl_3$. Although GSH reacts with $COCl_2$ to form predominantly diglutathion-S-yl carbonate, it reacts with $COBr_2$ to form predominantly GSSG and presumably carbon monoxide (not yet identified).

Metabolism of carbon tetrachloride (CCl_4) - Rat liver microsomes in air convert CCl_4 to a metabolite that reacts with 2,6-dimethylphenol to form 4-chloro-2,6-dimethylphenol (4-ClDMP). The chlorine in the 4-ClDMP comes from CCl_4 and not from inorganic chloride. Thus 4-ClDMP cannot be formed by a chloroperoxidase reaction of cytochrome P-450 or by a reactive metabolite that readily exchanges

with inorganic chloride, such as hydrochlorous acid or chlorine gas. Although molecular orbital calculations (G. Loew, Stanford International) suggest the formation of Cl_3COCl as a plausible intermediate, the finding that high oxygen concentrations inhibit the reaction suggests the view that CCl_4 is first reductively cleaved to trichloromethyl free radical ($Cl_3C\cdot$) which reacts with oxygen to form trichloromethyl superoxide ($Cl_3CO_2\cdot$). Moreover, the latter mechanism is consist with our inability to detect an electrophilic chlorine generated from hexachloroethane.

Chloramphenicol metabolism - Liver microsomes from rats treated with phenobarbital convert chloramphenicol in air to p-nitrobenzyl alcohol and N-(2-hydroxyethyl) dichloroacetamide. The primary alcohol group of chloramphenicol is oxidized to an aldehyde and the resulting chloramphenicol aldehyde undergoes an aldo cleavage to form p-nitrobenzaldehyde and N-(2-oxo ethyl) dichloroacetamide. p-Nitrobenzaldehyde is reduced to p-nitrobenzyl alcohol by a reductase other than alcohol dehydrogenase.

Under nitrogen, chloramphenicol undergoes reductive cleavage to monodeschloro-chloramphenicol presumably through the formation of a radical which may be trapped by N-t-butylphenyl nitrone. During this reaction cytochrome P-450 is destroyed by an unexplored mechanism.

Bromobenzene metabolism - Several years ago, our Laboratory established that bromobenzene causes liver necrosis through the formation of a chemically reactive metabolite, thought to be bromobenzene-3,4-oxide. Radiolabelled bromobenzene, however, also was covalently bound to proteins in lung and kidney. Since treatment of rats with phenobarbital increased the covalent binding in the extrahepatic tissues without increasing the formation of reactive metabolites of bromobenzene by these organs *in vitro*, it seemed likely that epoxides were formed in the liver and transported to the extrahepatic organs. Recently, it was shown that p-bromophenol, a product of bromobenzene-3,4-oxide, may be metabolized to chemically reactive metabolites by microsomes from liver, lung, and kidney of rats. Since covalent binding of radiolabeled p-bromophenol can be inhibited by epoxide hydrolase, catechol-O-methyl transferase, superoxide dismutase and ascorbic acid, p-bromophenol is probably converted to an epoxide that rearranges to p-bromocatechol which in turn undergoes autoxidation. Although p-bromophenol administered to rats becomes covalently bound to liver, kidneys and lungs, it does not decrease the GSH concentration in liver or increase serum glutamate-pyruvate transaminase (SGPT) even at doses equivalent to toxic doses of bromobenzene. Thus the toxicity of bromobenzene in rats is entirely due to bromobenzene-3,4-oxide. Nevertheless, bromocatechol in mice increases SGPT activity and causes single cell necrosis in liver.

Several years ago, our Laboratory discovered treatment of rats with 3-methylcholanthrene decreased the toxicity of bromobenzene by shifting the metabolism from bromobenzene-3,4-oxide to o-bromophenol. Although the mechanism of o-bromophenol formation remains uncertain, it may not be as innocuous as once believed, because o-bromophenol in mice pretreated with phenobarbital followed by diethylmaleate causes kidney damage as assessed by an increase in BUN.

Pharmacokinetic Studies

Long-lived unstable metabolites. As pointed out in last year's report, it is-

important to determine whether the toxicity will be restricted to those cells in which the metabolite is formed or whether virtually every cell in the body must be considered as a potential target cell. To this end we are developing techniques to determine whether chemically reactive metabolite escape cells in which they are formed. In our basic approach, precursors of the chemically reactive metabolites are incubated with hepatocytes and various amounts of a nucleophile (such as proteins) that cannot rapidly enter cells but trap the metabolite leaving the cells. Using bovine serum albumin, we demonstrated that bromobenzene-3,4-oxide could leave hepatocytes, but were unable to estimate how much of the metabolite left the cells. During the past year, we found that in the absence of added glutathione, glutathionyl transferase B (GT-B also known as ligandin) was a better nucleophile than albumin. When GT-B was incubated with bromobenzene and liver microsomes, it was not inactivated. Thus the bromobenzene-3,4-oxide became covalently bound at sites other than the catalytic site of the enzyme. With graphical procedures we invented, we estimated that an infinite concentration of GT-B added to hepatocytes would have covalently bound about 35% of the total amount of bromobenzene, metabolized by the hepatocytes. We also calculated that virtually all of the glutathionyl metabolites and intracellular covalently bound metabolites were formed as the reactive metabolites generated in the endoplasmic reticulum passed to the medium but that virtually all the other metabolites were formed from the reactive metabolites after they left the cells. The relative contributions of the various glutathionyl transferases in hepatocytes to the formation of the two glutathionyl conjugates formed from bromobenzene-3,4-oxide remains unclear. All of the transferases (GT-A, GT-B, GT-C and GT-D + E) catalyze the formation of both glutathione derivatives but the relative rates of formation of the two derivatives differ with the enzyme. Only the GT-D + E preparations provides a ratio that approximates that found in bile, (4-S form/3-S form) = 1.6.

Furosemide - Several years ago, our Laboratory found that the dose threshold in the hepatotoxicity and covalent binding of furosemide in livers of mice was due to a proportional increase in furosemide metabolism as the dose was increased. Subsequently, the dose threshold was attributed by Spitznagel et al. to the saturation of the biliary transport of furosemide. But this explanation would be inconsisent with the current view of the biliary transport mechanisms. A reinvestigation of the problem revealed that increasing the dose of furosemide caused a greater decrease in the renal clearance than in the biliary clearance. Moreover, preliminary studies on the covalent binding of furosemide to mouse hepatocytes have failed to reveal the presence of an outward transport system. The dose dependent increase in the toxicity and metabolism of furosemide is probably due to a decrease in renal clearance of furosemide caused by saturation of a renal transport system, kidney damage or both.

Desipramine - Because there can be marked genetic differences in the metabolism of drugs, many laboratories have begun to search for polymorphisms in the cytochrome P-450 enzymes. In collaboration with the Clinical Psychobiology Branch (NIMH) we have discovered that slow hydroxylators of desipramine are apparently more prevalent among Chinese than Caucasians.

Multiforms of Cytochrome P-450

Liver microsomes contain at least 12 cytochromes P-450, each one of which may

metabolize a given drug at different rates to different metabolites. Moreover, inducers may increase the synthesis of more than one form of cytochrome P-450. Because of this complexity, new strategies will be needed to estimate the relative amounts of the various cytochromes P-450 present in patients and their substrate specificites. To this end, we are developing an approach designed to discover whether a given antibody preparation combines with only one functional form of cytochrome P-450. The approach is based on the realization that when a single enzyme converts a drug to several metabolites, the apparent K_m value will be the same for the formation of all the metabolites regardless of the mechanism of the enzyme. Thus the relative rates of formation of the metabolites will be independent of the concentration of the substrate. Moreover, even in different enzyme preparations the relative rates of formation of the metabolites should be the same unless the preparations contain substances that cause allosteric changes at the catalytic site of the enzyme. Such changes, however, would be revealed as "functionally different enzymes". With this approach, we are evaluating a monoclonal antibody developed by H. Gelboin (NCI) which reacts with cytochrome $P\text{-}450_c$, an isozyme induced by 3-methylcholanthrene and β-naphthoflavone (βNF) in rats. Using R-propranolol as a substrate, we have found that the antibody reacts with βNF inducible forms of cytochrome P-450 in liver microsomes from rats, mice, guinea pigs and rabbits but not hamsters. Since the ratios of rates of formation of the metabolites inhibited by the antibody differed with the animal species, however, the antibody apparently combines with different forms of cytochrome P-450 having similar antigenic sites. It thus seems likely that some monoclonal antibodies developed with cytochrome P-450 purified from livers of animals may cross react with human cytochromes P-450 and thus may be useful in studies of the substrate specificities of the cytochrome P-450 in human tissues. Indeed, Gelboin has shown that the antibody cross reacts with a human placental cytochrome P-450.

Mechanisms of Toxicity

Effects of Ca++ on toxicity - Others have postulated that toxicants may cause liver necrosis by increasing the permeability of the cell membranes to Ca++. However, we have shown that the cytotoxicity caused by acetaminophen does not require the transfer of extracellular Ca++ into cells. Instead, Ca++ exerts two opposing actions on the release of lactate dehydrogenase (LDH). After preincubation of hepatocytes with acetaminophen, Ca++ hastens the rate of release of LDH but decreases the amount of LDH ultimately released into the medium. Moreover, decreasing the pH of the medium delays the release of LDH into the medium. Electron microscopy revealed a marked heterogeneity in the sensitivity of the cells; many cells appeared normal while others were completely lysed.

Effects of thyroidectomy on acetaminophen metabolism - Hepatocytes from thyroidectomized male rats were smaller than normal. Nevertheless, the conversion of acetaminophen per cell to acetaminophen glucuronide was increased, and to acetaminophen sulfate and glutathionyl acetaminophen were deceased. Studies with cell free systems indicated that the decrease in the formation of acetaminophen sulfate was not due to decreases in transferase activity. Instead it was due to decreases in PAPS synthesis, possibly caused by impairment of sulfate transport into hepatocytes. By contrast, the decrease in the formation of glutathion-S-yl acetaminophen is apparently due to a loss of a high affinity form of cytochrome P-450.

Toxicity in cultured cardiomyocytes - We have been able to culture cardiomyocytes that in the presence of 1.0 μM epinephrine beat rhythmically and synchronously. Both adriamycin (14-hydroxydaunomycin) and daunomycin caused cell damage, but daunomycin was about 3 to 4 times more toxic. Although these drugs are thought to evoke their toxic effects through the formation of superoxide and lipid peroxidation, daunomycin did not increase malonaldehyde formation, a decomposition product of peroxidized lipid. The mechanism of the cardiotoxicity thus remains obscure.

Role of Histamine in Physiologic and Pathologic Reactions

A large segment of the population undergoes some type of allergic reaction during the year and for some individuals the consequences may be severe or fatal. Our studies of the synthesis, storage and release of histamine from mast cells and inactivation of histamine by vascular endothelial cells are related to this problem. In addition histamine's role in gastric secretion continues to be an active area of research in the Section of Cellular Pharmacology.

Our past studies have shown that intact mast cells take up histamine, decarboxylate it and incorporate the newly formed histamine into granular stores. Histamine synthetic activity is largely lost upon cell disruption and for this reason we have continued studies with isolated intact mast cells. Our success this year in separating rat peritoneal mast cells according to their state of maturity has enabled us to show that the rat peritonium contains mitotically active precursor cells and a mixture of immature, mature and degenerating mast cells. During maturation, the histamine synthetic activity, which is high initially, declines markedly and histamine content increases from less than 1 pg to over 12 pg/cell. Degranulation caused by compound 48/80 is negligible in the smaller immature cells, by increases progressively as the mast cells mature and then declines as the cells degenerate. Moreover, the reaction to antigen may also be dependent upon the maturity of mast cells in individual organs. It is also apparent that mast cell degranulation is best studied with purified mature cells instead of the heterogenous populations of mast cells used previously.

Collaborative studies with workers at the V.A. Hospital in Los Angeles have shown that gastric mucosal histamine is localized in an enterochromaffin-like cell in rodents and mast cells in dog and other species. We have described in detail the biochemical morphological characteristics of these cells but so far have found no gastrin or cholinergic receptors on them nor have we been able to identify mediators of histamine release. We have previously shown that the acid-producing parietal cells possess functional histamine receptors (H_2) and high histamine methyl transferase activity.

Histamine is cleared rapidly from the blood passing through the stomach and other organs. Our finding that microvascular endothelial cells possess histamine-inactivating enzymes point to this cell as a possible site of inactivation. These cells also contain a compound 48/80 resistant pool of histamine which may be sustained by continuous histamine synthesis. Significance is attached to this finding because of the old (but unsubstantiated) theory that histamine is a regulator of the microcirculation.

Pathways of Inflammation and Their Suppression by Drugs

We have continued to characterize the various patterns of inflammatory response

in the rat pleural cavity to delineate the roles played by different mediators. It is now evident that multiple mechanisms exist for both increased vascular permeability and white cell infiltration. The partial degranulation of mast cells after dextran injection, for example, leads to rapid effusion of a protein-like fluid whereas more extensive degranulation of mast cells by anti-IgE or compound 48/80, leads to rapid effusion of fluid and plasma protein. Clearly a single process, e.g. opening of capillary pores, cannot acount for both of these responses. Evidence that histamine is responsible for these early responses is that they are suppressed by H_1 and H_2 histamine antagonists, but not by indomethacin. Neutrophil infiltration is observed at later times (4 hr) following the injection of anti-IgE but, for reasons unclear to us, not after injection of dextran or compound 48/80. The neutrophil chemoattractant has not been identified but the leukotrienes or chemotactic factors in the mast cell are possible candidates that will be further investigated.

In contrast to mast cell degranulation, quite different responses are evoked by carrageenan or zymosan which are believed to activate the complement system. There is no immediate fluid effusion or mast cell degranulation but after a delay of 1 hr there is a progressive accumulation of fluid, plasma proteins, neutrophil chemotactic activity and neutrophils. The initial event appears to be leakage of plasma proteins into the extravascular space and then generation of chemotactic factors(s) possibly from plasma complement components. The leakage of protein (and as a consequence generation of chemotactic activity) is suppressed by indomethacin, which suggests that products of cyclooxygenase catabolism mediate this process.

It is apparent that alternate pathways exist for fluid extravasation and neutrophil infiltration. Since no drug is likely to act equally well on all the pathways, our long term goal is to identify the pattern of mediator release and an effective drug therapy for each type of inflammatory response.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00617-06 LCP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

The mechanism of carrageenan induced inflammation in rat

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Theresa N. Lo	Research Chemist	LCP	NHLBI
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COOPERATING UNITS (if any)
None

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SECTION
Cellular Pharmacology

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Chemotactic activity in carrageenan (0.5 mg)-induce pleural exudates was demonstrated by the chemotactic response of neutrophils in the modified Boyden chamber. Activity was proportional to exudate protein in the range of 0.75 to 1.5 mg protein per chamber (1.2 ml). The activity differed from that in rat serum or plasma in that it was stable for 30 min at 56°C. Indomethacin (5 mg/kg i.v.) reduced equally protein content (56%) and total chemotactic activity (58%); i.e., chemotactic activity/mg of exudate protein was unchanged. Intrapleural injection of autologous or homologous serum induced infiltration of neutrophils; and when injected in two doses to maintain protein levels comparable to those found with carrageenan, the number of neutrophils in the exudates were also comparable. In contrast to the neutrophil response to carrageenan, the response to serum was not inhibited by indomethacin. The results suggest that in the carrageenan model, indomethacin acts primarily by inhibiting plasma protein exudation and thereby, the availability of plasma precursors of chemotactic factor(s).

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: Our earlier work on pathways underlying carrageenan-induced inflammation indicates that the response is of slow onset. Mast cells remain intact and there is progressive accumulation of fluid, plasma protein, and neutrophils over the course of 2-8 hr. Carrageenan is a high molecular weight sulfated polysaccharide. It is not in itself chemotactic for neutrophils but is believed to activate endogenous precursors of chemotactic activity. Although there is evidence that activation of the complement system mediates the recruitment of neutrophils in response to a variety of high molecular weight substances (e.g., zymosan, bacterial lipopolysaccharides) and that metabolites of arachidonic acid contribute to this process, there is little specific data on the mode of action of carrageenan.

Because fluid and white cell infiltration are suppressed in a dose-dependent manner by indomethacin or other nonsteroidal anti-inflammatory drugs the cyclooxygenase pathway of arachidonic acid metabolism appears to be involved but it is not clear whether the primary effect of this drug is suppression of plasma exudation or generation of chemotactic factor(s). We have assessed the relationship of plasma protein exudation, generation of chemotactic activity, and neutrophil accumulation after carrageenan injection and the effects of indomethacin treatment on these parameters. On the assumption that chemotactic activity was derived from plasma protein precursor, the effects of homologous and autologous plasma and serum injected into the pleural cavity were tested. The data suggest that the generation of chemotactic activity is dependent on availability of plasma protein and that indomethacin's primary action may be suppression of exudation of plasma protein.

Methods Employed: Purification of neutrophils from rat blood. Samples of heparinized rat blood were obtained by cardiac puncture. Neutrophils were purified by the procedue of Boyum (Scand J. Clin. Lab. Invest. 21: 77, 1968). To increase the percentage of circulation neutrophils from 12 to over 50%, carragneen an (0.5 mg) was given by intrapleural injection and rats killed 3 hr later (Almedia et al., J. Pharmacol.Exp.Ther.214:74,1980). Blood obtained from 3 such rats yielded 1.1 x 10^6 leukocytes (> 90% viability) of which 75-96% (mean 82%) were neutrophils.

Preparation of neutrophils from pleural exudates. Carrageenan was injected intrapleurally into 3 rats and the pleural fluid collected 3 hr later. The exudate and washings were pooled. The cells were washed three times and suspended in a Gey's-HEPES (25 mM), BSA (2% w/v) solution (2.5 x 10^6 cells/ml) to use in assays of chemotactic activity. More than 90% of the cells were neutrophils and viability (determined by the fluorescein diacetate/ethidium dibromide staining procedure) exceeded 95%.

Preparation of plasma and autologous and homologous serum. For the preparation of autologous serum, a butterfly needle was inserted into the tail vein and blood was collected in a polypropylene vial. For the collection of homologous serum or plasma, rats were anesthetized with ether. The chest

cavity was opened and blood was drawn from the right ventricle into a dry or heparinized polypropylene syringes. In some experiments, rats were given heparin (60 units/kg i.v.), the carotid artery was cannulated with polyethylene tubing and blood was collected in heparinized containers. To obtain serum, blood was kept at room temperature for 30 min and then centrifuged at 1300 x g for 10 min. (All centrifugations were carried out at 4°C). For plasma, heparinized blood (5 units/ml blood) samples were centrifuged at 28,000 x g for 20 min. Where indicated, samples were "heat-treated" by immersion in a water bath (56°C ± 0.2°C) for 30 min; all others were stored on crushed ice until used.

Injection of drugs and agents: collection of exudation and preparation of exudate extracts. Carrageenan (0.5% w/v) and indomethacin solutions were prepared as previously described. Autologous serum (0.3 to 0.5 ml) was diluted with normal saline to give a final volume of 1 ml. Homologous serum, prepared from pooled sera (cardiac puncture), was diluted with normal saline to a concentration of 55 mg of protein/ml. Rats were given vehicle or indomethacin (5 mg/ kg) intravenously. Thirty min later they were lightly anesthetized with ether, carragenan (0.1 ml) or diluted serum (1 ml) was injected into the pleural cavity. At timed intervals, rats were killed by ether, the exudate was aspirated into a plastic syringe, and the cavity was washed with 1 ml of the Gey's-HEPES buffer. The exudate and washings were pooled. The volume reported was corrected by subtracting the 1 ml wash. Total and differential cell counts were determined by standard techniques described in our previous publication. The remainder of the exudate was centrifuged at 600 x g for 10 min at 4°C. Samples of the supernatant fluid were assayed for protein by the method of Lowry and for chemotactic activity as described below.

Assay of chemotactic activity. A modified Boyden Chamber with a micropore filter of (3-μm diameter pores) was used. The upper compartment contained 0.2 ml of cell suspension (500,000 cells). The lower compartment contained 1.2 ml of the cell-free fraction of the pleural exudate, sera or plasma. These were diluted with Gey's-HEPES buffer to give the protein concentration indicated (mg/1.2 ml chambers). The chambers were incubated for 90 min and the filters stained with hematoxylin. Migration of neutrophils was assessed by the "microscopic sectioning" technique of Maderazo and Woronick (Clin. Immunol. Immunopathol. 11,196,1978) in which the average distance migated (Locomotion Index) by neutrophils is assessed. These procedures were described in detail in last year's report.

Major Findings: Evidence for the presence of chemotactic activity in carrageenan-induced exudate and effect of indomethacin on generation of this activity. The presence of chemotactic activity was evident from the enhanced migration of neutrophils (obtained from peripheral blood or carrageenan exudates) in response to small amounts of cell-free pleural exudates. Typical values (mean LI ± SE, n= 6) obtained for cell migration were: 21.8 ± 0.4 for medium alone; 27.7 ± 1.8, 30.9 ± 0.8, and 37.7 ± 1.2 for samples of exudate containing 0.75, 1.0, and 1.5 mg exudate protein, respectively. A significant linear correlation between values for LI and mg of protein

was observed. The specific chemotactic activity, i.e., LI/mg protein of cell-free exudates obtained from 10 control and 10 indomethacin-treated rats was similar when the values for LI were calculated from the regression lines for each exudate (6.2 ± 0.9 mg protein for control versus 6.8 ± 0.7/mg protein for indomethacin-treated rats, n = 10). Indomethacin treatment (5 mg/kg i.v.) reduced the accumulation of cells (-41%), volume of exudate (-47%), total protein (-56%), and total chemotactic activity (-58%) to similar degrees. Thus, while the specific chemotactic activity was unchanged, total chemotactic activity generated was reduced in proportion to protein content.

Evidence for the plasma protein origin of the neutraphil chemotactic activity. In all experiments exudate protein content and cell count were highly correlated ($p<0.001$ R 0.95). The protein concentration ranged from 40-80% of that in plasma and this correlation was unchanged in indomethacin treated rats. All major classes of plasma proteins including complement component Δ5 were identified by the double immunodiffusion (Ouchterlony) technique. Preliminary filtration studies suggest that the chemotactic factors is of high molecular weight, but potential artifacts (e.g. binding) have not been ruled out at this time.

Comparison of chemotactic activities in cell-free exudates, serum, and plasma. Neutrophils exhibited chemotaxis towards serum, plasma, as well as cell-free exudates. The chemotactic activities of all three fluids were of similar magnitude when assayed with 1.5 mg or less protein per chamber. The chemotactic activity of plasma and serum was destroyed completely by heating at 56°C for 30 min. Only a small loss (< 20%) of activity was observed upon heating exudates and this loss was apparent only when exudate was assayed in concentrations of 1.5 and 3.0 mg protein/chamber. The data suggest, therefore, a loss of heat labile factor and appearance of a heat stable factor once plasma protein reached the extravascular space.

Inflammatory response to intrapleural injection of autologous and homologous serum and effect of indomethacin. A significant portion (38 to 71%, mean 57%) of the injected serum protein was lost from the pleural cavity during the course of 4 hr. A second injection of serum after 2 hr resulted in an enhanced migration of neutrophils. The numbers of white cells were correlated with the amount of residual serum protein and no difference was observed in this regard, whether autologous or homologous serum was used or single or two injections of serum were given. In one experiment where two injections of serum were given, the magnitude of response was similar to that produced by carrageenan.

Prior injection of indomethacin appeared to have little effect on the response to serum. No significant difference was observed in the number of white cells in control and indomethacin-treated rats.

Significance to Biomedical Research and the Program of the Institute. Our studies indicate that carrageenan-induced inflammation is a useful model to study role of mediator(s) other than histamine and mast cell derived products. We have shown that increased vascular permeability to plasma

protein is associated with the production of chemotactic factor(s) and neutrophil accumulation and that indomethacin and other nonsteroidal anti-inflamatory drugs inhibit primarily plasma protein exudation and secondarily generation of plasma protein derived chemotactic factors.

Proposed Course of Project: Further studies will focus on questions as to 1) whether increased vascular permeability is mediated by arachidonic acid metabolites (as suggested by the studies of indomethacin), 2) the characteristics and identity of chemotactic factor(s) in plasma and exudate and the possible role of the complement system in the generation of these factors. The latter study will include molecular weight determination, use of antibodies to C5a and effect of heat treatment.

Publications:

Lo, T.N., Almedia, A.P. and Beaven, M.A.: Dextran and carrageenan evoke different inflammatory responses in rat with respect to composition of infiltrates and effect of indomethacin. J. Pharmacol. Exp. Therap. 221: 261-267, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00619-06 LCP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Role of histamine in gastric secretion

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Michael A. Beaven	Deputy Chief	LCP	NHLBI
OTHER: Diane Aiken	Biologist	LCP	NHLBI

COOPERATING UNITS (if any)
Drs. Andrew H. Soll, and Jon Isenberg, Center for Ulcer Research and Education, Wadsworth V.A. Hospital, Los Angeles, CA. and Gastroenterology Division, La Jolla V.A. Hospital, La Jolla, CA.

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SUMMARY OF WORK (200 words or less - underline keywords)

Studies with highly purified preparations of cells from collagenase digests of gastric mucosa suggest that in some species histamine (and histidine decarboxylase) is located in mast cells which lay in close proximity to the parietal cells. In rodents, these substances are present in enterochromaffin-like (ECL) cells which are embedded in the pit of the gastric gland. The major histamine metabolizing enzyme activity in dog gastric musoca is histamine methyltransferase which is located in the parietal cell and in rat, diamine oxidase (location undetermined). Conditions for a short term culture of the histamine containing cell have been established but, so far, histamine release has not been observed with a variety of gastric hormones and neurotransmitters. Studies in patients with duodenal ulcers have shown that following an infusion of histamine, the amine is cleared less rapidly from the circulation than in normal subjects.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: The finding that the histamine H_2 receptor antagonists block gastric secretion in a variety of species provided direct evidence that histamine has an essential but undefined role in gastric secretion. The separation of different cell populations in gastric mucosa of several species by Soll and Grossman at the Center for Ulcer Reseach and Education (CURE), Wadsorth V.A.Hospital, Los Angeles, and their subsequent collaboration with us has allowed us to identify the sites of histamine synthesis, storage and metabolism within gastric mucosa (see conclusions). In this report, we describe studies that 1) complete our characterization of the histamine-containing cells in rat and dog gastric mucosa; 2) tests for putative mediators of histamine release in short-term cultures of isolated gastric mast cells; and 3) were designed to establish the relationship between plasma histamine level and gastric secretory response in control subjects and patients with duodenal ucler. The latter study has indicated that these patients may have a reduced ability to clear histamine from the circulation.

Methods Employed: 1) Studies in isolated cell systems. Gastric mucosa and other tissues were treated sequentially with collagenase EDTA, and then a second time with collagenase. The digests were passed through an albumin density gradient to separate histamine, parietal and chief cells. Each of these cell fractions was further fractionated by use of the Beckman elutriator. Details of these procedures are now published (Gastroenterology 77, 1283, 1979; 80,717,1981; 82, 254, 1982). This year further refinements of the protocols have been made to 1) allow better separation of the different cell types and 2) reduce the time required for cell isolation. The procedures are time consuming and the introduction of simple single step separation procedures has made it possible to obtain fractions of cells of reasonable purity (40-70%) in less than half a day (These studies were carried out by Dr. Soll at CURE). Meanwhile we have improved the separation procedure for mast cells as descibed elsewhere.

Rat peritoneal mast cells and dog gastric mucosa mast cells were dispersed onto coated (gelatin, fibrin, polylysine) plates and incubated in media supplemented with various peptide hormones and antibiotics. The cells were harvested 48 and 72 hr later. Sterile equipment was used for these experiments (these studies were peformed at cure by Dr. Soll).

Cell viability was monitored by microscopy and dye exclusion tests. In studies performed at CURE, the fractions were divided into portions of about 10^5 cells, and duplicate sets were shipped to the NIH.

Samples were assayed for DNA, histamine, serotonin and the various enzyme activities by the microassay procedures developed in this Laboratory and described in previous project reports. Decarboxylase activities were assayed with 0.25 mM substrate.

2) Assay of plasma histamine levels in volunteers and patients with duodenal ulcer. Eighteen patients with dudoenal ulcer were studied during inactive asymptomatic phase of their disease. These patients were matched by age with healthy control subjects. All subjects were studied twice after an overnight fast. Gastric juice was collected through a nasogastric tube. Histamine or gastrin was infused into a small vein in the dorsal side of one hand. Blood was collected from the opposite arm through a scalp vein needle inserted into an arm vein. Saline was infused for 30 min folowed by histamine in doses of successively 1.25, 2.5, 5, 10, 20 and 40 µg/kg/hr. Each dose was infused for 30 min in increasing stepwise doses. On the day gastrin was infused, saline followed by gastrin in doses of 6, 12.5, 25,50, 100 and 200 pmoles/kg/h were infused in a similar stepwise fashion. Gastric juice was collected for each 30 min period and blood (5 ml) was collected at minus 30, 0 and at 30 min intervals thereafter to coincide with the end of each infusion period. Blood was also collected at 1, 2.5, 5, 7.5, 10, 15 and 30 min after stopping the infusion. Blood was allowed to clot to obtain sera. Sera and gastric juice was assayed for histamine (at NIH) and gastrin (at Wadsworth V.A. Hospital). The protocol (P.I. Jon Isenberg) was submitted to Human Subjects Committee, Univ. of California, San Diego.

For the purposes of this study the radioenzymatic histamine assay (Anal. Biochem. 94,425,1979) was modified substantially to improve the sensitivity of the assay. Plasma samples (10 µl) were incubated (37°, 90 min) with 30 µl of reagent which contained 1 µCi ^{3}H-labeled S-adenosyl-L-methionine (Sp. Act. 15 Ci/mmole), purified rat kidney histamine methyltransferase (4 µg protein), bovine serum albumin (1 mg) in 50 mM sodium phosphate buffer, pH 7.9. The reaction was terminated by the addition of perchloric acid and unlabeled methylhistamine followed by 10 N sodium hydroxide as described previously. The labeled product, N-methylhistamine was extracted into a mixture of toluene/isoamyl alcohol (80:20). The organic phase was evaporated to dryness at room temperature. The residue was dissolved in 40 µl 0.1 N HCl and the extraction step repeated. The final extract was then assayed for radioactivity. Typical values obtained by this procedure were 229 $\pm$ 23 (0); 304 $\pm$ 6 (2.5); 382 $\pm$ 10 (5.5); 539 $\pm$ 15 (11.1) cpm (Values in parenthesis are pg histamine) and 400-600 cpm for sera from untreated subjects.

Major Findings. 1) Studies with freshly isolated cells and cells held in short-term culture (with Dr. Soll). Studies with isolated cells from dog liver and gastric mucosa and rat gastric mucosa have now been completed. The following account is a final summary of our past and recent findings.

In the dog tissues the histamine-containing cells were identified as mast-cells with a histamine content of 1.9 - 2.9 pg/cell compared to 14 pg histamine/rat peritoneal mast cell. With intact mast cells, the rate of histamine synthesis (pmoles/hr/10^{6} cells) was estimated to be 12 and 48 for mast cells from dog mucosa and liver and 400 for rat peritoneal mast cells. Synthetic activity was largely destroyed upon disruption of mast cells from rat and dog. Although a soluble decarboxylase activity could be detected in extracts of all these cells only that in peritoneal mast cells has been studied in detail and identified as histidine

decarboxylase activity. In dog mast cells the enzyme activity has been tentatively identified as histidine decarboxylase on the basis of its inhibition by α methylhistidine and lack of inhibition by ℓ methyl DOPA and the absence of DOPA decarboxylase activity. The dog mast cells had the characteristic morphology of mast cells (Gastroenterology 82: 254, 1982). Those in gastric mucosa, to our surprise, were devoid of gastrin and cholinergic receptors (unpublished).

The histamine-containing cell in rat gastric mucosa was shown to be an enterochromaffin-like (ECL) cell (see ZO1-HL-00619-05 CM) which differ from the mast cell both in its morphology and biochemical characteristics. These cells contain histamine (2-6 pg/cell) and abundant histidine (110-130 pmoles/hl10^6 cells) and DOPA (1100-2600 pmoles/hr/10^6 cells) decarboxylase activity. The two activities could be distinguished by use of specific inhibitors (i.e. ℓ methyl DOPA) and lack of competition between L-histidne and L-DOPA. The ECL cells possessed no IgE receptors and did not release histamine in response to compound 48/80. Light and Electron micrographs of the cells have been published (Gastroenterology 80,717,1981).

Cultures of dog liver and gastric mucosal mast cells remain viable and retain their histamine for up to 48 hr. As reported last year these cells showed no response (histamine release) to a variety of gastric hormones and neurotransmitters or after passive sensitization with serum from dogs sensitized to bovine serum albumin or ascaris antigen. This year histamine release was observed in liver mast cell cultures with 1:400 to 1:120 dilutions of antibody raised to rat IgE. It appears that dog liver mast cells possess IgE receptors and that anti-rat IgE antibody has sufficient cross reactivity to dog IgE to induce histamine release.

2) Comaprison of the effects of histamine and gastrin infusion in duodenal ulcer patients and normal volunteers (with Jon Isenberg). Studies were conducted to compare the gastric secretory response of normal subjects and patients with duodenal ulcer to histamine and gastrin. Although there are reports that these patients are more sensitive than normal subjects to infusions of gastrin and pentagastrin (J. Clin. Invest. 55,330, 1975; Gastroenterology 72, 1087, 1977) the effect of histamine has not been evaluated. From the viewpoint of our section, we felt that with no change in the experimental protocol additional useful information would be obtained if serum histamine levels were monitored since there are no data on the relationship between plasma histamine levels and rates of gastric secretion.

The actual changes in histamine levels were found to be quite small and considerable time was spent to increase the sensitivity of our assay. All steps in the assay were studied in detail and, as a consequence, the procedure was modified substantially. The procedure is sufficiently sensitive to assay 2.5 pg histamine per 10 µl sample.

In both patients and normal subjects serum histamine levels were 1.0 ± 0.1 ng/ml (range 0.5 to 1.2 ng/ml). In normal subjects, significnt increases were observed only with the highest doses of histamine (1.6 ± 0.3 and 2.4 ± 0.3 ng/ml with respectively 20 and 40 µg/kg/hr) whereas patients' plasma histamine levels increased with each dose of histamine (to 2.5 ± 0.3 ng/ml with the higher dose of histamine). After the infusion, histamine levels in normal subjects returned to normal within 2.5 min, but in patients histamine levels subsided slowly and did not return to normal until 10 min. The differences between the two groups were highly significant.

Significance to Biomedical Research and Program of the Institute: Our studies over the past 5 years now provide a clear picture as to the location of histamine, its sites of synthesis, metabolism and action within the gastric mucosa. In dog (and man) the gastric histamine stores and histidine decarboxylase are localized in mast cells which lie in the lamina propria adjacent to the parietal cells. The parietal cells contain receptors (H_2) for histamine and high histamine methyltransferase activity. The sites of histamine action and degredation are, therefore, in close proximity. In rat and possibly mouse (see last year's report), gastric histamine and histidine decarboxylase are located exclusively in an APUD-type cell that is now identified as an enterochromaffin-like (ECL) cell. These cells are not in the lamina propria but are embedded in the base of the gastric pits and it is thought that histamine diffuses into the lamina propria before reaching the parietal cell. Diamine oxidase is the sole catabolic enzyme in rat gastric mucosa but the location of this enzyme has not been identified. So far we have failed to identify possible mediator(s) of histamine release.

The studies in duodenal ulcer patients point to a possible defect in their ability to metabolize histamine. As histamine is thought to be a common mediator of gastric secretion, the high gastric secretory response (and ulcer formation) might be attributable to retarded destruction of histamine.

Proposed Course of Project: We will continue to focus studies on possible mechanisms of histamine release from histamine-containing cells by due of short-term culture experiments. Additional studies with duodenal ulcer patients may be undertaken pending further discussion with Dr. Isenberg.

Publication:

Beaven, M.A., Soll, A.H. and Levin, K.: Histamine synthesis by intact mast cells from canine fundic mucosa and liver. Gastroenterology 82:: 254-262, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00620-05 LCP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Regulation of Histamine Synthesis and Release in Tissues

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Michael A. Beaven	Deputy Chief	LCP	NHLBI
OTHERS:	Elizabeth Wolde-Mussie	Staff Fellow	LCP	NHLBI
	Diane Aiken	Biologist	LCP	NHLBI

COOPERATING UNITS (if any)
Dr. Andrew H. Soll, Wadsworth V.A. Hospital, Los Angeles, CA.

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Cellular Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	0.3	0.7

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies in which rat peritoneal mast cells were separated by elutriation into successive fractions of increasing cell size and maturity revealed that immature mast cells (Stages I and II) were found to have high histamine synthetic activity and low (<1 pg/cell) histamine content. With cells of increasing size and maturity synthetic activity decreased 90% and histamine content increased to 12 pg histamine/cell as did the number of intracellular granules. A gradation in response to compound 48/80 was also observed: small mast cells (Stage I) were resistant, those of larger size (Stage II) were partially responsive and large mast cells (Stages III and IV) were fully responsive to compound 48/80. Although mature mast cells released 68-83% of their granular histamine content in response to compound 48/80, intracellular histamine synthetic activity was unchanged. From our biochemical and histological studies, we conclude that during mast cell maturation there is a progressive increase in granule numbers and granule constituents (heparin and histamine), an increase in responsiveness of the cells to compound 48/80 and a decline in extragranular histamine synthetic activity.

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Project Description:

Objectives: Histamine is found in mammalian tissues in amounts ranging from less than 0.1 µg/g in blood to over 50 µg/g in gastric mucosa and lung and 1 mg/kg in mastocytomas. The enzyme responsible for histamine synthesis, histidine decarboxylase (EC 4.1.1.22), has been identified in extracts of many tissues. Paradoxically, histidine decarboxylase activity in extracts of most tissues is low and is insufficient to account for the high histamine levels, found in most tissues.

Last year, we presented evidence that cell disruption results in destruction of a carrier mediated histidine transport system (identified as system 'N'), which may serve to concentrate histidine within the cell, and in the inactivation of histidine decarboxylase probably through the action of proteolytic enzymes. For this reason, we have continued our studies of histamine synthesis in intact cells. We have examined the changes in histamine synthetic activity, histamine content and responsiveness to degranulating agents during the growth cycle of the rat peritoneal mast cell. Cells were isolated from the rat peritoneum, separated by size and examined for morphological and biochemical changes.

Methods Employed: Collection and Purification of Cells. Male, Sprague-Dawley rats (250 to 300 g) were killed by decapitation, and 10 ml of Hanks' balanced salt solution was injected into the abdominal cavity. The abdomen was then massaged for 90 sec, the abdominal wall was opened, and the fluid removed by syringe. The peritoneal cells were washed and resuspended in 10 ml of the Hanks' solution.

The cell suspension (150 x 10^6 cells) was loaded into an elutriator rotor (Beckman Instruments, Spinco Division) and separated into 12 fractions (100 ml) by successive increases in flow rate. Cell counts and size analysis were performed using a Particle Data Counter. The viability of cells was assessed by ethidium dibromide and fluoroescein diacetate dyes, light and electron microcopy.

The fractions were stored at 4°C in a refrigerator in sterile capped tubes. On the day of elutriation (day 0) and successive days thereafter (days 1,2,3, etc.), 0.5 ml aliquots were removed from them for preparation of slides and biochemical measurements as outlined below.

Histological studies. Slides were prepared by cytocentrifuge (Shandon Scientific Corp., London), air dried and stained with Wright's stain in an automatic stainer (Hema-Tek slide stainer, Ames Co., of Miles Laboratories, Elkhart, IN). For other stains, slides were fixed in Newcomer's fixative (30 min) rehydrated in ethanol/water mixtures (95,70 and 60% ethanol) and stained with Alcian blue followed by safranin (both dyes in 0.27 phosphate buffer adjusted to pH 1 with HCl) as described by Combs et al. (J. Cell Biol. 25:577,1965). The slides were dehydrated with ethanol (70, 95 and 100% ethanol), cleared with xylene and permanent mounts made of them. Mast cells were categorized into their stages (I,II,III and IV) of growth (300 cells examined on each slide) according to the histochemical criteria of Pretlow and Cassady (Am. J. Pathol. 61:323,1970).

Histamine release studies. Cells were recovered by centrifugation (100 g x 10 min) washed once with a Hanks'-HEPES (10 mM) bovine serum albumin (BSA) (0.1%) medium and resuspended in the same medium to give 2 x 10^6 cells/ml aliquots (95 µl) of this were incubated (37°) for 10 min in polypropylene vials and compound 48/80 (in 5 µl) was then added to make a final solution of 10 µg/ml. The suspensions were incubated for an additional 15 min. They were then centrifuged (100 g x 10 min). Both cell pellets and supernatant fluid were frozen and stored at -20°C.

Assay of histamine synthetic (intact cells) and histidine decarboxylase (cell extacts) activities. Cells were dispersed in Hanks' salt solution containing 10 mM HEPES buffer, pH 7.4 (0.5 x 10^6 cells/200 µl). Where noted, cells were disrupted by sonification (Kontes Ultrasonicator, maximum setting) for 10 sec. Intact and disrutped cells were incubated with β-^{3}H-(side chain carbon)-L-histidine (20 nCi and ^{14}C-carboxyl-labeled L-histamine (20 nCi). After trapping the $^{14}CO_2$ in Hyamine, the reaction mixture was assayed for ^{3}H-histamine by isotope dilution derivative analysis. These assays were described in last years report. The final concentration of histidine was 1 x 10^{-5}M.

Assay of histamine. For most samples, the enzymatic isotopic assay described previously (Anal. Biochem. 94, 425, 1974) was used. For samples of low histamine content, the assay described in report no. Z01 HL 00619-06 LCP was used. The latter assay was sufficiently sensitive to study histamine from single mast cells.

Major Findings: 1) Relationship between mast cell size, histamine level and histamine synthetic activity. Elutriation of rat peritoneal cells resulted in two peaks of cells. The larger one (mainly monocytes) appeared in fractions 2 to 5, the minor one (mast cells) appeared in fractions 8 to 11. Mast cells were, however, present in all fractions between 2 to 12 and it appeared that in each successive fraction there was a progressive increase in size and maturity (see below). The diameter of the cells varied from 10 µm (fraction 3) to 22 µm (fraction 12), although the majority of cells (> 85%) were 15 µm or greater in diameter.

The smaller mast cells possessed high histamine synthetic activity (200-300 pmoles/hr/10^6 cells) and low histamine content (< 1pg/cell). With increasing cell size, histamine content increased to reach a maximum of 12 pg/cell (fractions 8 and 9) and synthetic activity decreased to 20 pmoles/hr/10^6 cells. Likewise histidine decarboxylase activity recovered in cell extracts declined from 7 to 1 pmoles/hr/10^6 cells (i.e. greater than 95% loss of activity upon cell disruption).

2) Morphology of mast cells in elutriated fractions. Mast cells in Stage I of development were present largely in fractions 3 to 5; Stage II in fraction 4-6; Stage III in fractions 6 to 10; and Stage IV in fractions 8 to 12. Stage I cells possessed large nuclei with indications of mitotic activity and a few alcian blue staining granules (i.e. nonsulfatated mucopolysaccharides were present). Stage II cells possessed more granules some of which stained blue and some red (i.e., heparin was present) whereas Stage III cells contained many more

granules most of which stained red. In Stage IV cells all stained red and their numbers were such to obscure the nuclei. These data indicated that with increasing size, the number of granules and their heparin content increased progressively. The largest cells (fractions 10-12) showed reduced viability when compared to cells in fractions 6-8 (75% versus 95% viability).

In young rats (70 g), the total number of mast cells and the percentage of those that were mature (Stage IV) were much lower than those observed in old rats. The histamine synthetic activity and histamine content were otherwise similar to that seen in preparations from older rats.

3) Retention of histamine synthetic activity upon mast cells degranulation. Exposure of mature mast cells (fractions 9 and 10) to compound 48/80 resulted in substantial release of histamine (73-95%) but little or no loss of synthetic activity (n=6). No histidine decarboxylase activity was observed in the incubation medium. These data indicate that histamine is not synthesized in the granular matrix but at some extragranular site.

4) Response of mast cells in different elutriated fractions to compound 48/80. Mast cells in fraction 4 were resistant (less than 10% release), in fractions 5 and 6 partially resistant (28 and 56% release respectively) and fractions 7 and 8 fully responsive to compound 48/80 (79 and 78% release). Factions thereafter, showed a diminished response (70 down to 60%), but when values were corrected for the percentage of nonviable mast cells, the responses were similar to that observed with fraction 7 and 8.

Preliminary studies in which mast cells were diluted to give an estimated single cell in each aliquot suggested that it is possible to measure histamine release from single cells with our assay procedure.

5) Stability of elutriated mast cells upon storage at 4°. The number of viable mast cells and their responsiveness to compound 48/80 decreased progressively upon storage. The changes were at a slower rate (half-life 4-5 days) than we had expected, and experience has shown that the fraction can be used for up to 3 days after their separation. Fractions of small cells, however, lost their viability more rapidly than did large cells.

Significance to Biomedical Research. The time course of changes in histamine synthetic activity and histamine content during mast cell maturation has not been studied previously. The studies indicated that the mast cells are generated, and probably degenerate, within the peritoneal cavity. The peritoneal mast cells are not, therefore, a homogenous population and the marked changes in responsiveness to compound 48/80 indicate that studies of histamine release mechanisms with such mixed populations may be uninterpretable. As the present studies indicate, mature mast cells can be purified by elutriation. The ability to measure histamine release in single mast cells will provide a potentially useful approach to study mechanisms of release (i.e. whether or not release is a graded or all or none phenomenon). Because of the wide occurrence of immediate hypersensitivity reactions in man, and the potent effects of mast cell constituents on the cardiovascular and pulmonary systems,

an understanding of mast cell development and degranulation mechanisms is of clinical significance and is consistent with the mission of this Institute.

Proposed Course of Project: The time course of changes in histamine synthesis and histidine uptake following mast cell degranulation will be studied in short-term culture to see if histamine stores are replenished and how this process is affected by suicide inhibitors of histidine decarboxylase such as ℓ-fluoromethylhistidine. Studies of histamine release in single mast cells will be continued with special emphasis on the role of calcium in this process.

Publications:

Beaven, M.A., Roderick, N.B., Shaff, R.E. and Soll, A.H.: Histamine synthesis in the intact and disrupted rat mast cells. Biochem. Pharmacol. in press.

Beaven, M.A.: Factors regulating availability of histamine at tissue receptors. In Ganellin, C.R. and Parsons, J. (eds.): Pharmacology of Histamine Receptors. John Wright and Sons Ltd., London, in press.

Beaven, M.A.: Anapylactoid reactions to anesthetic drugs. Anesthesiology 55: 3-5, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 00631-02 LCP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Presence of Histamine and its Metabolizing Enzymes in Microvascular Endothelial Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Audrey Robinson-White	Guest Worker	LCP	NHLBI
OTHER:	Michael A. Beaven	Deputy Chief	LCP	NHLBI

COOPERATING UNITS (if any)

Dr. Robinson-White is a recipient of an NRSA postdoctoral fellowship.
Dr. Gordon Kauffman, Wadsworth V.A. Hospital and UCLA, Los Angeles, Ca.

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SECTION
Cellular Pharmacology

INSTITUTE AND LOCATION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Purified microvascular endothelial (MVE) cell preparations from guinea pig fat pad, heart, and brain were found to contain the histamine degrading enzymes, histamine methyltransferase (HMT) and diamine oxidase (DAO). The enzyme content of these cells accounted for most of the HMT activity in the whole fat pad, and a significant part of the enzyme activity in heart and brain. High HMT activity was also found in heart myocytes and gastric parietal cell, which like the capillary endothelial cell are important sites of histamine action. In addition to the histamine degrading enzymes the MVE cells contained a small pool of histamine that was resistant to compound 48/80 and appeared to turn over at a rapid rate. The data indicate that MVE cells contain a nonmast cell store of histamine as well as histamine inactivating enzymes.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: This project reflects our past interest in the phylogenetic origin and distribution of histamine histidine decarboxylase (HbD), histamine methyltransferase (HMT) (EC 2.1.1.8), and diamine oxidase (DAO) (EC 1.2.6). Our previous studies showed that histamine first appeared in brain and gastric mucosa of primitive vertebrate species and then in mast cells in terrestrial vertebrates. (Brain Research 208, 1249, 1981; Monogr. Allergy Vol.13, 1978). Histamine is thought to have a physiological role in brain and in gastric mucosa and, in higher vertebrates, a immunopathologic role when released from mast cells. This project is concerned with how histamine is disposed of after its release by physiological or immunological stimuli.

Earlier studies from this and other groups have shown that upon intravenous injection of labeled histamine, most (>80%) of the label can be recovered as labeled metabolites within 2 min in all major organs. When infused through isolated organs (heart, lung, kidney, hind limb and liver) or into the whole animal (see this report) histamine is cleared upon passage across all the major vascular beds. Since the vascular endothelial cell in lung and other tissues take up and inactivate serotonin and catecholamines it seemed a likely possibility that histamine was also degraded by these cells. Last year we reported (Reports No. Z01 HL 00631-01 CM) that isolated microvascular endothelial cells from various tissues of rat and guinea pig contain HMT and DAO activity and a small but significant pool of histamine. This year we have expanded this project to include studies of the uptake, turnover and efflux of histamine in these preparations and of the clearance of histamine across various vascular beds in dog.

Methods Employed: 1) Isolated cell preparations: Microvascular endothelial cells from brain cortex, fat pad and heart myocardium as well as myocytes and adipocytes were prepared and characterized as described in last year's report. Isolated rat kidney tubules (medulla and cortex) and glomeruli were prepared by the method of Scholer and Edelman (Amer. J. Physiol. 237, 350, 1979).

All preparations were assayed for viability, histamine content, and HMT, DAO and HdO activities by procedures outlined in last year's reports.

2) Measurement of histamine uptake, efflux and release. Uptake was measured in suspensions of isolated endothelial cells (50 µg of cell protein) in 100 µl Hanks' balanced salt solution which contained 10 mM HEPES, 500 µCi β-^{3}H-histamine and unlabeled histamine as indicated. The suspensions were incubated at 37°C (0° for blanks) for 15 min and then diluted with ice cold Hanks' solution (400 µl) to terminate the reaction. The suspensions were centrifuged in a Beckman Microfuge for 1 min, the supernatant fractions

were discarded and the pellets were resuspended in 100 µl of ice cold media. Fifty µl of each suspension was saved for the assay of protein and 50 µl was centrifuged (microfuge) for 1 min through 250 µl of a mixture of dioctyl and dibutylphthalate mixture and the cell pellets recovered for the assay of radioactivity. Preliminary studies had indicated that there was little carry over of extracellular lable (< 100 dpm ^{3}H) and no significant loss of cellular histamine by this procedure.

Efflux of unlabeled and labeled histamine was determined in both normal cells and cells previously loaded with β-^{3}H-histamine (60 min preincubation with 500 µCi labeled histamine). All cell preparations (2 mg protein) were washed twice with 5 ml Hanks' medium before measurement of efflux. Cell suspensions (50 µg protein/100 µl) in Hanks were incubated at 37° over different time periods (generally 15,30,45,60,90 and 120 min) at 37° and then centrifuged (microfuge) for 1 min. The supernatant fraction was removed, a portion of this and the cell pellet was saved for the assay of histamine and protein. In some experiments, L-histidine, ℓ-fluoromethyl-histidine and Brocresine were added to the cell suspension.

Histamine release was studied by the above procedure except that compound 48/80 (10 µg/ml final concentration) was added to one set of tubes and vehicle to another (controls). The tubes were incubated for 15 min at 37° and processed as described above.

All the above determinations were done in quadruplicate and calculations were based on the protein content of (Lowry) of each tube.

3) Measurement of histamine clearance of histamine across vascular beds in dog. Histamine was infused (160 µg/kg/min) for 15 min into the cephalic vein of an anesthetized dog in which nonoccluding catheters had been placed into various blood vessels (experiment performed by Dr. Gordon Kauffman). Plasma samples were withdrawn at different times during and following the period of infusion. The samples were assayed for histamine by radioenzymatic assay as described in report no. Z01 HL 00620-05 LCP.

Major Findings: 1) Enzyme activities in microvessel and other cell preparations per each tissue. Enzyme activities have now been measured in a total of 4-10 preparations. In guinea pig microvascular endothelial cells from fat pad, myocardium and brain cortex, HMT activity was respectively 4.3 ± 0.6, 4.4 ± 0.9 and 1.2 ± 0.4 units/mg protein (1 unit = 1 nmole, histamine methylated /hr in the presence of 1 µM histamine) compared to values of 0.3 ± 0.1 and 1.0 ± 0.2 for guinea pig adipocytes and myocytes, 2.8 ± 1.5 for rat kidney tubules and 3.4 for dog gastric parietal cells. The last two were chosen for comparison as we have found them to contain the highest HMT activity of all tissues surveyed to date (Anal. Biochem. 94, 425, 1979; Gastroenterology 82, 254, 1982). No HMT activity (i.e. <0.01 units/mg protein) was observed in any of the rat microvascular preparations; trace activity (0.02-0.1 units/mg protein) in myocytes; and as mentioned above activity was observed in rat brain (0.34 ± 0.03 units/mg protien); ileum (1.6 ± 0.3) and kidney (12.3 ± 0.9).

DAO activities in microvessel preparations from rat and guinea pig varied from 1.5 to 4.1 units/mg protein (1 unit = 1 pmole histamine deaminated/hr in the presence of 0.1 μM histamine) and were 4 to 40 times those in the whole tissue from which the microvessel had been obtained.

2) Uptake, synthesis and turnover of histamine in vascular endothelial cells. All microvascular cell preparations contained an intracellular pool of histamine of approximately 10 ng/mg cell protein (200 ng/g tissue). Both uptake of extracellular histamine and synthesis of histamine was observed with these preparations.

Histamine was taken up by a temperature dependent process. Similar studies with rat neutrophils, red blood cells, mast cells and mixed peritoneal cells indicate that histamine uptake was slower than that observed in the endothelial cell preparations (e.g. 4-6 versus 14-22 pmoles/hr/mg protein with 1 μM histamine) and was by a nonsaturable process.

Histamine synthesis was not observed in disrupted cell preparations, but in suspensions of intact cells intracellular histamine content remained unchanged with time (up to 120 min) whereas the histamine content of the media increased and by 60-90 min equaled that in the cells (4 experiments). In one experiment, the addition of Brocresine (10^{-5}M) on fluoromethylhistidine (10^{-5}-10^{6}M) supressed this increase with no change in the intracellular histamine levels. Whether the intracellular pool is sustained by uptake and synthesis and is inactivated by the cellular HMT or DAO activity is uncertain until studies with labeled histidine and histamine are complete. It is clear that if the synthesized histamine enters the intracellular pool, turnover of this pool would be rapid.

The intracellular histamine content was not decreased by exposure to compound 48/80 (10 μg/ml). In the endothelial cell preparation studied to date (rat fat pad, myocardium, cortex and guinea pig fat pad) the maximum decrease was observed in rat fat pad endothelial cells (50 ± 9 versus 44 ± 9 ng histamine/mg protein n = 5). In contrast, a 75 ± 5% (range 46-91%, n = 12) decrease in histamine content was observed with rat peritoneal mast cells.

3) Clearance of histamine across various vascular beds in dog. Constant intravenous infusion of histamine for 15 min resulted in equilibration of plasma histamine levels throughout the circulation. At this point histamine levels in the right atrium and carotid artery were similar (35-41 ng histamine/ml plasma). There were, however, marked differences in levels in various parts of the venous system. These ranged from 0.2 ng/ml in the femoral vein to 25 ng/ml in the jugular vein. The estimated extraction ratios across the hind limb, kidney, intestine, stomach, liver and head were respectively 99.9, 90,78,71,65 and 39%. The lower clearances across the visera and brain may indicate that some histamine escaped degradation by passage through arteriovenous shunts. Once infusion had stopped, histamine levels in the right atrium declined rapidly (half-life of 25 secs.) to normal levels (0.2 to 2.5 ng/ml).

Significance to Biomedical Research and the Program of the Institute: All microvascular endothelial preparations studied so far contain significant histamine-degrading activity. The ability of these cells to take up histamine may facilitate access of circulating histamine to degrading enzymes and might account for the rapid clearance from the circulation. Since small increases (less than 5 ng/ml) in plasma histamine are associated with widespread urticarial and cardiovascular reactions in man and increases of 10-20 ng/ml are observed during severe anaphylactic shock, the presence of HMT and DAO activity at critical sites would be essential for survival. Of clinical concern, a wide range of drugs (antihistamines, antimalarials, local anesthetics, histamine agonists) are potent inhibitors of HMT and, in some cases, DAO activity (see previous reports), and some of these drugs have been shown to potentiate histamine's actions in vivo and in vitro.

The present studies also indicate the presence in vascular endothelial cells of a nonmast cell store of histamine whose functional signifiance remains to be determined.

Proposed Course of Project: The uptake synthesis and degradation of labeled histamine and histidine by intact endothelial cells will be studied in conjuction with inhibitors of HMT and DAO. Initial experiments will be carried out with microvessels from fat pad as the uptake and degradation of serotonin have been previously studied in this tissue by the principal investigator (J. Pharmacol. Exp. Ther. 216:125, 1981).

Publications:

Beaven, M.A.: Histamine: Its role in physiological and pathological processes. Monographs in Allergy, Vol. 13, Japanese-Edition (revised and translated into Japanese) Rikogahussya and Co., Ltd., pp. 1-158, 1981 (originally published by S. Karger, Basel, p.1-114, 1978).

Beaven, M.A., Robinson-White, A., Roderick, N.B., and Kauffman, G.: The demonstration of histamine release in clinical conditions: A review of past and present assay procedures. Proceedings of the Symposium on Histamine and Antihistamines in Anaesthesia and Surgery, Munich, June 3-5, 1981. Klin. Wochesschrift, in press.

Robinson-White, A. and Beaven, M.A.: Presence of histamine, histamine methyltransferase and diamine oxidase ativity in rat and guinea pig microvascular endothelial cells. J. Pharm. Exptl. Therap., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00858-01 LCP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
The Relative Roles of Urinary and Biliary Excretion in Furosemide Hepatotoxicity in the Mouse

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Harriet M. Maling	Pharmacologist	LCP	NHLBI
Other:	James R. Gillette	Chief	LCP	NHLBI
	Wilford F. Saul	Chemist	LCP	NHLBI

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SECTION
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NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.4	0.7	0.7

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The hepatotoxicity produced by furosemide in mice was evaluated by levels of plasma glutamic-pyruvic transaminase (GPT). Doses of 200 mg/kg s.c. or less did not elevate plasma levels of GPT. In contrast, doses of 300,500, and 800 mg/kg increased plasma GPT greatly. Urine and bile were collected during successive 15- and 30-minute intervals for 180 minutes after injection of furosemide (50,200,300, or 500 mg/kg s.c.). Extrapolations to infinity were made on the basis of semi-logarithmic plots of excretion rates versus time and/or rectilinear plots of cumulative excretion versus time. About 23-33% of the injected furosemide was excreted unchanged in bile, at all doses tested. In contrast, the percent dose excreted unchanged in urine decreased steadily with increase in dose from 32.4% after 50 mg/kg to 15.9% after 200 mg/kg and 10.0% after 500 mg/kg. From about 40% after 50 mg/kg to about 66% after 500 mg/kg could not be accounted for by excretion in urine and bile and therefore probably represented metabolized furosemide. The impaired renal function and the increased metabolism are probably the major factors leading to hepatic necrosis after large doses of furosemide in the mouse.

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Project Description:

Objectives: The objective of this project is to evaluate the relative roles of biliary and urinary excretion in producing hepatic necrosis in the mouse.

Methods Employed: Male NIH GP mice, weighing 26-36 g, were anesthetized with sodium pentobarbital (ca. 75 mg/kg i.p.). The common bile duct was cannulated as described by Spitznagle et al (Toxicol. Appl. Pharmacol. 39:283-294, 1977). The gall bladder and distal end of the bile duct were ligated before cannulation of the common bile duct with a polyethylene (PE10) tube. The urinary bladder was cannulated with a PE50 tube, inserted through a small incision made with a scissors. Urine and bile were collected in preweighed 1.5 ml polypropylene Eppendorf micro-test tubes in successive 15-minute periods during the first hour and in 30-minute successive intervals during the second and third hours after the s.c. injection of furosemide (Lasix^{R}, Hoechst-Roussel Pharmaceuticals, Inc.). Concentrations of unchanged furosemide in bile and urine and in blood taken at the end of each experiment were measured by a sensitive HPLC method, using acetonitrile-0.01 M sodium acetate, pH 5.0, as the mobile phase (Lin, Smith, Benet and Hoener, J. Chromatogr. 163:315-321, 1979). Excretion rates for furosemide in bile and urine were plotted against time on semi-logarithmic paper. The total amounts excreted in bile and urine were plotted against time on rectilinear graph paper.

Major Findings: The percent dose of furosemide excreted unchanged in bile did not change appreciably from 26.9% after 50 mg/kg to 23.6% after 500 mg/kg. In contrast, the percent dose excreted in urine decreased with increase in dose from 32.4% after 50 mg/kg to 10.0% after 500 mg/kg. The amount of furosemide not excreted into urine and bile ranged from 40.7% after 50 mg/kg to 66.5% after 500 mg/kg. The unaccountable furosemide probably represents metabolized furosemide. The percent dose of furosemide measured in the carcass at 180 minutes was not sufficient to explain all the unaccountable furosemide, which was calculated as 100 - (% excreted in bile + % excreted in urine); biliary and urinary excretion were both extrapolated to infinity.

Significance to Biomedical Research and the Program of the Institute: This study was conducted in the mouse because large doses of furosemide produce liver necrosis in the mouse, but not in the rat. Excretion of furosemide in bile is clearly shown to be an important pathway of elimination, but does not account for the dose dependent increase in the conversion of furosemide to covalently bound metabolites.

Proposed Course of Project: A manuscript is now in preparation. A study is planned to measure covalent binding of ^{14}C-labeled furosemide to mouse hepatocytes in vitro.

Publications:

Arvidsson, A., Maling, H.M., Saul, W.F. and Gillette, J.R.: An evaluation of the effects of cephaloridine on urate excretion in the rat. Pharacology, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00859-01 LCP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Development of a new principle for estimating hepatic blood flow rates

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Harriet M. Maling	Pharmacologist	LCP	NHLBI
Other:	James R. Gillette	Chief	LCP	NHLBI
	Lance R. Pohl	Pharmacologist	LCP	NHLBI
	Wilford F. Saul	Chemist	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Physiology Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.3	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Theoretically, a system in which a drug is converted to a metabolite that in turn is both rapidly excreted into urine and metabolized to a second metabolite in liver may be used to estimate hepatic blood flow rates. A search of the literature has suggested that an appropriate precursor-drug-metabolite combination for estimating functional hepatic blood flow might be aspirin-salicylic acid-salicyluric acid. We plan to study (C-14)-labeled aspirin as the precursor and (H3)-labeled salicylic acid as the drug in rabbits, which normally excrete an alkaline urine. Alkaline urine is important because salicylate excretion in urine is much greater when the urine is alkaline than at acid pH.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: The objective of this project is to calculate hepatic blood flow from measurements confined to systematic blood samples and urine collected after a single rapid intravenous injection containing a ^{14}C-labeled precursor and a ^{3}H-labeled drug. We chose ^{14}C-labeled aspirin (acetylsalicylic acid) as the presursor and ^{3}H-labeled salicylic acid as the drug on the basis of the literature. Experiments are planned in rabbits because their urine is normally alkaline and salicylate excretion is known to be appreciably greater in alkaline urine than in acidic urine.

Major Findings: Aspirin and salicylic acid have many advantages for this study. The toxicities of both aspirin and salicylate are known to be low. The pharmacokinetics of salicylate in man have been published (Levy, G. Pharmacokinetics of salicylate in man. Drug Metabolism Reviews 9:3-19, 1979). Aspirin is rapidly deacetylated in the liver to salicylic acid, thus serving as a precursor of salicylic acid. Salicylic acid is excreted in the urine partly unchanged and partly conjugated either with glycine as salicyluric acid, or with glucuronic acid as salicyl phenolic glucuronide and salicyl acyl glucuronide. The amount excreted in urine as the oxidation product, gentisic acid, is too small to affect our pharmacokinetic analysis. All the metabolites of salicylic acid are excreted almost entirely in urine. In man, renal excretion of salicylic acid and the formation of salicyl acyl glucuronide and gentisic acid are first order processes. We do not know the kinetics of these processes in the rabbit.

Significance to Biomedical Research and Program of the Institute: This project should develop a relatively non-invasive procedure for estimating hepatic blood flow. It will test the theoretical relationships which were developed in a previous study (Z01 HL 00857-02 LCP).

Proposed Course of Project: The values of hepatic blood flow obtained in this procedure with a single rapid injection of ^{14}C-aspirin and ^{3}H-salicylic acid will be compared with the values calculated under steady-state conditions with an intravenous infusion of the same precursor and drug.

Publications:

Gillette, J.R., Saul, W.F. and Maling, H.M.: A new principle for estimating hepatic blood flow rates. Pharmacology 23:237-246, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00860-01 LCP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Studies on the metabolism of furosemide by mouse hepatocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Harriet M. Maling	Pharmacologist	LCP	NHLBI
Other:	James R. Gillette	Chief	LCP	NHLBI
	Wilford F. Saul	Chemist	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Physiology Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.1	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

When the rate of metabolism of a substance is diffusion limited, the pattern of metabolism may change with increasing concentrations of the substance even when its total metabolic clearance is not changed. The objective of this project is to determine whether the metabolism of furosemide by mouse hepatocytes is diffusion limited. The covalent binding of (C-14)-labeled furosemide to isolated hepatocytes will be measured as an index of metabolism. Experiments are now in progress to adapt the procedures described by other investigators for the preparation of rat hepatocytes to the preparation of mouse hepatocytes.

32f

Project Description:

Objectives: The objective of this project is to determine whether the metabolism of furosemide by mouse hepatocytes is diffusion limited. Covalent binding of ^{14}C-labeled furosemide to mouse hepatocytes will be measured as an index of metabolism. Plans are in progress to utilize mouse hepatocytes rather than rat hepatocytes because large doses of furosemide produce hepatotoxicity in the mouse but not in the rat.

Methods Employed: Experiments are in progress to modify the procedures described by Seglen for the preparation of rat hepatocytes (P.O. Seglen, Exper. Cell Res. 74:450, 1972; 76:25, 1973; 82:391, 1973) to the preparation of mouse hepatocytes. This procedure involves an initial perfusion through the portal vein with a Ca^{2+}-free buffer during which the liver is removed, followed by a perfusion in vitro with a collagenase- and Ca^{2+}-containing buffer. After perfusion for about 20 min, the liver is transferred to a Petri dish and the cells are dispersed by gentle manipulation. The crude cell suspension is purified by 3 or 4 low speed centrifugations, which separate the parenchymal cells from debris and non-parenchymal cells.

Major Findings: The preparation procedure is still being modified, to increase yield and viability. At present, our yield of mouse hepatocytes is about 10^7 cells and suspensions of mouse hepatocytes show a viability of about 80%, calculated on the basis of trypan blue exclusion.

Significance to Biomedical Research and the Program of the Institute: Studies of the metabolism of furosemide by mouse hepatocytes should give insight into the mechanisms for the threshold dose for the hepatotoxicity of furosemide.

Proposed Course of Project: The metabolism of furosemide by mouse hepatocytes will be compared with the metabolism by rat hepatocytes, to determine whether there is a species difference in hepatocyte metabolism corresponding to the susceptibility to hepatotoxicity in the mouse and the lack of hepatotoxicity in the rat.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00932-03 LCP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

New Metabolites of Chloramphenicol

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Patricia Morris	Chemist	LCP	NHLBI
Other:	Lance R. Pohl	Pharmacologist	LCP	NHLBI
	John W. George	Chemist	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.9	0.2	0.7

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Hepatic microsomal cytochromes P-450 of rats treated with phenobarbital metabolized chloramphenicol (CAP) aerobically to p-nitrobenzyl alcohol, N-(2-oxoethyl)-dichloroacetamide, N-(2-hydroxyethyl)-dichloroacetamide, and CAP aldehyde (CAP with the primary alcohol group oxidized to an aldehyde group). The first step in the formation of these products is the oxidation of CAP to CAP aldehyde. This β-hydroxyaldehyde can undergo either spontaneously or catalytically a retro-aldo-scission to p-nitrobenzaldehyde and N-(2-oxoethyl) dichloroacetamide. These intermediates are rapidly reduced by a NADPH or NADH dependent reductase to p-nitrobenzyl alcohol and N-(2-hydroxyethyl)-dichloroacetamide respectively. Under conditions of low oxygen tension, cytochromes P-450 catalyzes a reductive dechlorination of CAP into a monochloro-radical intermediate. This reactive species can either abstract a hydrogen atom from the medium to yield deschloro-CAP or react covalently with microsomal protein. Since cytochrome P-450 is destroyed during this sequence of reactions it is possible that it is a major site of covalent interaction. The destruction of cytochrome P-450 may explain how CAP alters the metabolism and toxicity of a variety of compounds.

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PHS-6040
(Rev. 2-81)

Project Description:

Objective: We have previously reported that rat liver cytochrome P-450 cleaves a carbon-carbon bond of CAP aerobically and activates CAP into a reactive intermediate anaerobically. These metabolic reactions have now been further characterized.

Methods Employed: CAP labeled with ^{14}C in the dichloroacetamide carbons and ^{3}H in the benzylic position was incubated with liver microsomes from phenobarbital pretreated rats. After 30 min., the reactions were stopped by the addition of methanol. The methanol supernatants were analyzed by HPLC and the metabolites were identified by mass spectrometry. Covalent binding to microsomal protein and cytochrome P-450 levels were determined by standard methods.

Major Findings: Under aerobic conditions liver microsomes from rats treated with phenobarbital convert CAP to seven metabolites as detected by HPLC. Five of these metabolites have previously been identified as dichloroacetic acid, CAP base, CAP oxamic acid, p-nitrobenzyl alcohol, and N-(2-hydroxyethyl)-dichloroacetamide ($HOCH_2CH_2NHCOCHCl_2$).

The remaining two metabolites have now been identified as N-(2-oxoethyl)-dichloroacetamide ($OCHCH_2NHCOCHCl_2$) and CAP aldehyde (CAP with the primary alcohol group oxidized to an aldehyde group) by mass spectrometry. Their formation was dependent upon the presence of NADPH and O_2 and was inhibited by SKF 525-A or CO/O_2 (8:2, v/v). Moreover, the metabolites could not be detected when the incubations were performed with liver microsomes from untreated rats or rats treated with β-naphthoflavone.

When synthetic CAP aldehyde was incubated in buffer, it was found to undergo a spontaneous retro aldo cleavage to p-nitrobenzaldehyde and N-(2-oxoethyl)-dichloroacetamide which were rapidly reduced by sodium borodeuteride to monodeuterated p-nitrobenzyl alcohol and N-(2-hydroxyethyl)-dichloroacetamide respectively. Moreover, p-nitrobenzaldehyde was rapidly reduced to p-nitrobenzyl alcohol by rat liver microsomes aerobically in the presence of NADH or NADPH. The alcohol dehydrogenase inhibitor, pyrazole, only inhibited the reaction marginally.

As previously reported, CAP was metabolized anaerobically by rat liver cytochrome P-450 predominantly to deschloro-CAP and a covalently bonded product. The free radical trap reagent, N-tertiary-butyl-phenyl nitrone has now been shown to decrease the formation of both of these products by approximately 50 percent. In addition, CAP was found to cause a 32 percent reduction in the level of cytochrome P-450 when it was incubated anaerobically with rat liver microsomes.

Significance to Biomedical Research and the Program of the Institute: The results of the present study show that rat liver cytochromes P-450 induced by phenobarbital catalyze an unusual cleavage of a carbon-carbon. This reaction appears to be a stepwise process which involves the initial oxidation of the primary alcohol group of CAP to an aldehyde. The resulting product is a β-hydroxyaldehyde which can either spontaneously or possibly enzymatically undergo a retro-aldo cleavage to p-nitrobenzaldehyde and N-(2-oxoethyl)-dichloroacetamide. If cytochrome P-450 catalyzes the retro-aldo cleavage step, this

would be a property of the enzyme not previously recognized. Moreover, since the ß-naphthoflavone-induced and the constituitive cytochromes P-450 did not catalyze the cleavage of the carbon-carbon of CAP, this catalytic property may be a unique property of a specific form of cytochrome P-450. By knowing the molecular basis of these differences, we should obtain a better fundamental understanding of the catalytic properties of cytochrome P-450.

The observation that pyrazole did not appreciably inhibit the microsomal reduction of p-nitrobenzyl alcohol to p-nitrobenzyl alcohol indicates that enzymes other than alcohol dehydrogenase catalyze this reaction. Since aldehydes are potential intermediate in the metabolism of many compounds and are relatively reactive and potentially toxic compounds, characterizing these enzymes should have general importance in drug metabolism and toxicology.

Cytochromes P-450 induced by phenobarbital also have the unique property of reductively dechlorinating CAP into a reactive intermediate. Since the radical spin trap reagent, N-t-butyl phenyl nitrone, blocked both the formation of deschloro-CAP and the covalently bonded product, it seems reasonable that a radical intermediate is involved in both of these metabolic pathways. A possible pathway explaining these results involves the initial one electron reduction of CAP by cytochrome P-450 into an anion radical intermediate. This species would be unstable and could spontaneously dechlorinate to give a monochloro radical intermediate that should either abstract a hydrogen atom from the media to produce deschloro-CAP or covalently bond to microsomal protein. Moreover, since 32 percent of cytochrome P-450 appears to be destroyed during the anaerobic activation of CAP, it is possible that the radical intermediate covalently binds to and inactivates cytochrome P-450. If this occurs, it could explain, at least in part, how chloramphenical alters the pharmacologic and toxicologic properties of other drugs and chemicals. Whether or not any of the new metabolites of CAP identified in this study are involved in the bone marrow toxicity produced by CAP remains to be determined.

Proposed Course of Project: We plan to 1) characterize how cytochromes P-450 catalyze reductive dehalogenation reactions; 2) determine how CAP destroys cytochrome P-450 and 3) investigate the potential bone marrow toxicity of metabolites of CAP. The results of such studies should help in the design of safer derivatives of CAP and other halogenated compounds used as drugs.

Publications:

Morris, P.L., Burke, T.R., George, J.W., and Pohl, L.R.: A new pathway for the oxidative metabolism of chloramphenicol by rat liver microsomes: Cleavage of Carbon-Carbon bond. Drug Metab. Disp. (in press).

Gross, B.J., Branchflower, R.V., Burke, T.R., Lees, D.E., and Pohl, L.R.: Bone marrow toxicity in vitro of chloramphenicol and its metabolites. Toxicol. Appl. Pharmacol. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00938-02 LCP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Mechanisms of formation of glutathione conjugates of bromobenzene

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Terrence J. Monks	Vist. Assoc.	LCP	NHLBI
Other:	James R. Gillette	Chief	LCP	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme-Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.25	0.25	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have previously isolated and identified two glutathione conjugates of bromobenzene as trans-3-bromo-6(glutathion-S-yl)-cyclohexa-2, 4-dien-1-ol (BB-GSHA) and trans-4-bromo-6(glutathion-S-yl)-cyclohexa-2, 4-dien-1-ol (BB-GSHB). The two conjugates are formed in unequal amounts in a ratio of BB-GSHA: BB-GSHB of 1.6:1. We have therefore purified the various forms of glutathione-S-transferase enzymes from rat liver and examined their ability to catalyze the reaction of bromobenzene-3, 4-oxide with glutathione. All forms of the enzyme isolated exhibit catalytic activity towards the epoxide. However, the ratio of the two conjugates formed with the purified enzymes varies from the ratio obtained both in vivo and with rat liver microsomes and 100,000 x g supernatant. The combined conjugates are metabolized by γ-glutamyl transpeptidase at apparently identical rates, and give rise to five products. One of the products is less water soluble than the starting substrates and might be the dehydrated, aromatized cysteinyl glycine conjugate.

PHS-6040
(Rev. 2-81)

Project Description:

Objective: The two bromobenzene-glutathione conjugates previously described by this laboratory are formed by rat liver microsomes and 100,000 x g supernatant, and excreted into bile in vivo, in unequal amounts. The present study was designed to investigate the biochemical mechanism responsible for the apparent stereospecifity in conjugate formation and/or excretion.

Methods Employed: The formation of the bromobenzene-glutathione conjugates has been shown to be dependent upon the presence of the glutathione transferase enzymes, present in the 100,000 x g rat liver supernatant. Thus the different forms of glutathione transferases were purified by published procedures to ascertain possible differences in their ability to catalyze the conjugation of glutathione with bromobenzene-3, 4-oxide. Such differences might explain the preferential addition of glutathione to the 4-position rather than the 3-position of the epoxide. Another possible reason for the difference in the amounts of the two conjugates might be in their susceptibility to further metabolism. Therefore the two conjugates were incubated with γ-glutamyl transpeptidase to investigate the above possibility.

Major Findings: Glutathione transferases A, B, C, and D + E, all catalyze the formation of bromobenzene-glutathione conjugates. However, transferases A, B, and C all produce more of BB-GSH B than BB-GSH A, in contrast to results obtained in vivo and in vitro with 100,000 x g supernatant. However, glutathione transferases D + E combined produce the conjugates in the same ratio as is observed in vivo and in vitro with 100,000 x g supernatant.

Incubation of the combined bromobenzene-glutathione conjugates with γ-glutamyl transpeptidase yields at least five products. The products have not been characterized. However, one of the products has a long retention time on HPLC and may be the dehydrated, aromatized cysteinyl-glycine conjugates. Moreover, the rate of metabolism of the two glutathione conjugates by γ-glutamyl transpeptidase was identical. The covalent binding of the 3,4-epoxide to the glutathione transferases does not affect their catalytic activity.

Significance to Biomedical Research and Program of the Institute: The differences in the rate of formation of the two bromobenzene-glutathione conjugates derived from bromobenzene-3, 4-oxide may have important implications. The rate of detoxification of chemically reactive metabolites is important in limiting their potential as toxic agents. Therefore elucidating and understanding those factors which influence the detoxification of reactive metabolites is of immediate importance.

Proposed Course of Study: We intend to characterize the products derived from the two bromobenzene-glutathione conjugates when incubated with γ-glutamyl transpeptidase. Moreover we intend to investigate further the discrepancy between the rates of formation of the two glutathione conjugates in vivo and in microsomes supplemented with 100,000 x g supernatant, and their formation in microsomes supplemented with purified preparations of the glutathione transferase enzymes.

Publications: Monks, T.J., Pohl, L.R., Gillette, J.R., Hong, M., Highet, R.J., Ferretti, J.A., and Hinson, J.A.: Stereoselective formation of bromobenzene glutathione conjugates. Chem. Biol. Int., (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00940-02 LCP

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Electrophilic halogens as potential toxic metabolites of halocarbons

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Bruce A. Mico	Guest Worker	LCP	NHLBI
Other:	Lance R. Pohl	Pharmacologist	LCP	NHLBI
	John W. George	Chemist	LCP	NHLBI
	Richard V. Branchflower	Staff Fellow	LCP	NHLBI

COOPERATING UNITS (if any)

Dr. Gilda Loew is in the Life Sciences Division, SRI International, Menlo Park, California. Dr. Robert Highet is in the Laboratory of Chemistry, NHLBI.

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Enzyme-Drug Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.65	1.45	0.20

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The formation of electrophilic chlorine from carbon tetrachloride is catalyzed by liver microsomal cytochrome P-450. Stable isotope studies demonstrate that chloride ion is not the source of the electrophilic chlorine but rather the chlorine atom trapped is derived from CCl4. These studies exclude a chloroperoxidase reaction as the mechanism of electrophilic chlorine formation. Molecular orbital calculations indicate that cytochrome P-450 triplet oxene is capable of directly oxidizing CCl4 to the halogenating agent trichloromethyl hypochlorite (CCl_3OCl). Experimental studies, however, indicate that the predominant mechanism involves the one electron reduction of CCl4 to trichloromethyl radical ($CCl_3\cdot$) which reacts with O2 to form trichloromethyl peroxy radical ($CCl_3OO\cdot$). Subsequent reactions of this intermediate leads to electrophilic chlorine. This reductive-oxygenation mechanism has important implications concerning the catalytic action of cytochromes P-450 and lipid peroxidation and toxicity produced by various xenobiotics.

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PHS-6040
(Rev. 2-81)

Project Description:

Objectives: The objective of this project is to determine the molecular mechanism by which electrophilic halogens are formed during the liver microsomal metabolism of tetrahalomethanes. These studies include: 1) characterizing the reaction as enzyme mediated and what enzymes are involved, 2) studying structure-activity relationships for electrophilic halogen formation, 3) applying molecular orbital calculations as a guide to reaction mechanisms and 4) studying the potential involvement of this pathway in the toxicity produced by halocarbon drugs and environmental chemicals.

Methods Employed: 2-Chloro-4,6-dimethylphenol was synthesized by the reaction of sulfuryl chloride with 2,4-dimethylphenol and its structure determined by 1H NMR and mass spectrometry. Liver microsomes were prepared from Sprague Dawley rats pretreated with phenobarbital. NADPH cytochrome P-450 reductase and cytochrome P-450 were purified by literature procedures. Unless indicated otherwise, substrates (5 mM) were incubated for 30 min with liver microsomes (2 mg/ml, 2 ml), NADPH (1 mM) and 2,6-dimethylphenol (1 mM). The formation of trapped electrophilic halogen as 4-halo-2,6-dimethylphenol was quantitated by capillary gas chromatography mass spectrometry in the selective ion monitoring mode. Various chemicals or enzymes were added to microsomal incubations as probes of the above objectives of the project. The assay procedure for 4-halo-2,6-dimethylphenol has been improved this year. An internal standard, 2-chloro-4,6-dimethylphenol has been incorporated into the assay procedure to control for losses during sample preparation. In addition a capillary gas chromatography column was adapted to the assay procedure. This has enhanced both sensitivity and resolution of the assay method.

Major Findings: The rate of formation of electrophilic chlorine from CCl_4 was linear with time of incubation (5-30 min) and microsomal protein concentration (0.25-2.0 mg/ml). The rate of electrophilic chlorine formation was saturable with increasing CCl_4 concentration and the Km and Vmax for this reaction were 0.98 mM and 1.28 nmole/4 mg microsomal protein/30 minutes, respectively. The formation of electrophilic chlorine during the microsomal metabolism of CCl_4 was inhibited by cytochrome P-450 inhibitors CO/O_2 (8:2) and piperonyl butoxide. With purified components of the mixed function oxidase system, the formation of electrophilic chlorine was dependent on all three components; lipid, NADPH cytochrome P-450 reductase and cytochrome P-450. In the absence of cytochrome P-450, no electrophilic chlorine was detected. In summary, these studies demonstrate that the formation of electrophilic chlorine from CCl_4 is catalyzed by cytochrome P-450.

The formation of electrophilic chlorine during the metabolism of CCl_4 was not due to chloride ion oxidation, since (1) no electrophilic chlorine was trapped from incubation mixtures containing microsomal protein, 2,6-dimethylphenol and sodium chloride with either NADPH, H_2O_2 or cumene hydroperoxide. (2) When (^{35}Cl) CCl_4 was incubated in the presence of (^{37}Cl) NaCl, the electrophilic chlorine atom trapped was exclusively ^{35}Cl. These data clearly excluded a chloroperoxidase reaction as the mechanism of electrophilic chlorine formation and indicate that cytochrome P-450 catalyzes a reaction with CCl_4 leading to carbon-chlorine bond cleavage and chlorine atom oxidation.

One possible pathway of electrophilic chlorine formation from CCl_4 involves direct oxidation of CCl_4 to trichloromethyl hypochlorite (CCl_3OCl). This reaction is without chemical or biological precedence. We therefore undertook theoretical calculations to determine whether this reaction is energetically feasible. Molecular orbital calculations indicate that cytochrome P-450 triplet oxene is capable of abstracting a chlorine atom from CCl_4. Recombination of $CCl_3\cdot$ and $\cdot OCl$ yields CCl_3OCl. Further calculations indicate that this intermediate contains an electropositive chlorine atom and can react with water to form phosgene and the halogenating agent hypochlorous acid. Similar calculations with $CHCl_3$ as the substrate indicate that hydrogen atom abstraction is greatly favored over chlorine atom abstraction. These results predict that $CHCl_3$ would not yield electrophilic chlorine. These theoretical results are consistent with the experimental observation that $CHCl_3$ or $CDCl_3$ do not yield electrophilic chlorine.

However, other pathways of CCl_4 activation are also possible. CCl_4 may first be reductively dehalogenated to trichloromethyl radical ($CCl_3\cdot$). Reaction of this radical with molecular oxygen, which is known to occur at diffusional rates, would form trichloromethylperoxy radical ($CCl_3OO\cdot$). This intermediate is implicated in the photooxidation of CCl_4 to phosgene and electrophilic forms of chlorine. This pathway may be termed as a reductive-oxygenation. Alternatively, CCl_4 may react with superoxide anion radical to form $CCl_3OO\cdot$ radical directly. These three possible mechanisms of CCl_4 activation, direct oxidation, reductive-oxygenation, and reaction with superoxide, have been differentiated in studies described below.

When NADPH was replaced by H_2O_2, cumene hydroperoxide, iodobenzene diacetate or sodium periodate in standard microsomal incubations of CCl_4 or CBr_4, no electrophilic halogens were observed. This result is inconsistent with a direct oxidation mechanism of CCl_4 activation. The rate of electrophilic formation during microsomal incubations with CCl_4 was not affected by addition of superoxide dismutase, catalase or sodium azide. These results suggest that neither superoxide, hydrogen peroxide or oxygen metabolites derived from H_2O_2 and superoxide are involved in CCl_4 activation.

Maximal rates of electrophilic chlorine formation were observed when microsomal incubations were conducted under atmospheres of 2-5% oxygen, whereas the reaction was markedly inhibited by 0% and 100% oxygen atmospheres. These results are consistent with a mechanism in which CCl_4 is reductively oxygenated to trichloromethyl peroxy radical. Subsequent reactions of this intermediate lead to electrophilic chlorine.

A survey of chlorinated or brominated halocarbons was made to study structure-activity relationships for electrophilic halogen formation. The results of this survey indicate that the formation of electrophilic halogen is limited to tetrahalomethanes. Notably, hexachloroethane did not yield electrophilic chlorine.

Significance to Biomedical Research and Program of the Institute: The results of these studies have several important implications in drug metabolism and toxicology. For example, our results better define the mechanism of action of cytochrome P-450. We have demonstrated for the first time that cytochromes P-450 can not catalyze oxidations of halide ions like chloroperoxidase but instead catalyze an unprecedented oxidation of a carbon halogen bond with concommitant formation of electrophilic halogen.

The first step of this reaction with CCl_4 appears to be a one electron reduction to an anion radical intermediate that spontaneously decomposes to $CCl_3\cdot$. Reaction of this radical with molecular O_2 yields $CCl_3OO\cdot$. Further reaction of $CCl_3OO\cdot$ either chemically or enzymatically mediated by cytochrome P-450 is believed to produce electrophilic chlorine (Cl^+) and $COCl_2$. $CCl_3OO\cdot$ might also abstract a hydrogen atom from unsaturated fatty acids and initiate lipid peroxidation. Therefore, the hepatotoxicity and possibly carcinogenicity of CCl_4 may be due to three reactive metabolites, namely $CCl_3OO\bullet$, $COCl_2$ and Cl^+.

It is important to emphasize that the results of these studies indicate that cytochrome P-450 can reduce compounds under normal oxygen tension. Previously it was generally believed that this enzyme only reduced compounds in the absence of oxygen. Therefore several reactions that were once thought to occur only at low oxygen tension such as nitro reduction, halocarbon reduction, azo reduction and N-oxide reduction might take place under normal oxygen tension, depending upon the oxidation potential of the particular compounds.

Proposed Course of Project: We plan to extend our studies on the mechanism of electrophilic halogen formation and its relationship to the toxicity of tetrahalomethanes. These studies include: 1) further characterization of the molecular events of electrophilic halogen formation, 2) determining if carbonyl halides are formed by the same pathway as electrophilic halogens, 3) determination of the chemical properties of the halogenating species and 4) determination of the relationship of electrophilic halogen formation to tetrahalocarbon hepatotoxicity.

Publications:

Mico, B.A., Branchflower, R.V., Pohl, L.R., Pudzianowski, A.T. and Loew, G.H.: "Oxidation of carbon tetrachloride, bromotrichloromethane, and carbon tetrabromide by rat liver microsomes to electrophilic halogens." Life Sciences 30, 131-137 (1982).

Mico, B.A. and Pohl, L.R.: "Metabolism of carbon tetrachloride to electrophilic chloride by liver microsomes: Exclusion of a cytochrome P-450 catalyzed chloroperoxidase reaction." Biochem. Biophys. Res. Comm. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00941-02 LCP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Effects of Ketones on Cytochromes P-450

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Richard V. Branchflower	Staff Fellow	LCP	NHLBI
Other:	Lance R. Pohl	Pharmacologist	LCP	NHLBI
	John W. George	Chemist	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme-Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.65	0.45	0.20

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Several alphatic ketones to which humans are exposed are known to potentiate the toxicities produced by various xenobiotics including CHC13 and CC14. We have purified to apparent homogeneity the major form of cytochrome P-450 induced by methyl-butyl ketone (MBK) and demonstrated that it is similar to the major form induced by phenobarbital (PB). They have the same apparent molecular weights, are digested similarly by chymotrypsin and V-8 protease, and both catalyze the metabolism of CHC13 to COC12. Initial studies with acetone demonstrate that it also increases cytochromes P-450 levels and results in an increased rate of microsomal metabolism of CHC13 to COC12. Therefore, MBK and acetone appear to potentiate the hepatotoxicity of CHC13 and possibly other compounds by increasing the rate of their metabolism to toxic metabolites.

PHS-6040
(Rev. 2-81)

Project Description:

Objective: The goal of this project is to determine whether ketones potentiate the toxicity of xenobiotics by inducing the synthesis of specific forms of cytochromes P-450 that catalyze their metabolism to toxic metabolites.

Methods Employed: Microsomes, cytochromes P-450, DE-52 anion exchange chromatography, the assays of the glutathione adduct of phosgene and warfarin metabolites, SDS polyacrylamide electrophoresis, peptide mapping and the determination of glutathione levels were all performed by published methods.

Major Findings: Liver microsomes from phenobarbital (PB) and methyl butyl ketone (MBK) treated rats metabolized warfarin to a similar pattern of 4', 6, 7 and 8 hydroxy metabolites. The major form of liver microsomal cytochromes P-450 from PB and MBK pretreated rats was purified to apparent homogeneity by fractional precipitation with polyethylene glycol and DE-52 anion column chromatography. Both preparations ran as single bands on SDS polyacrylamide electrophoresis (MW = 48,000 daltons) as did a mixture of the two preparations. Incubation of the proteins with V-8 protease or chymotrypsin resulted in essentially identical peptide maps. Both purified proteins were capable of metabolizing $CHCl_3$ to phosgene ($COCl_2$) in the presence of NADPH-cytochrome P-450 reductase and dilaurylphosphatidyl choline.

Initial studies on the mechanism of potentiation of $CHCl_3$ toxicity by acetone showed that 3 days of acetone treatment increased the level of liver cytochromes P-450 1.4 fold and the metabolism of $CHCl_3$ to $COCl_2$ by rat liver microsomes 1.7 fold as compared to untreated rats. Under these same conditions, MBK treatment increased the level of cytochromes P-450 1.3 fold and the metabolism of $CHCl_3$ to $COCl_2$ 3.6 fold. Acetone treatment, unlike that with MBK, did not produce any discernible effects on the DE-52 anion exchange chromatogram of rat liver cytochromes P-450.

Significance to Biomedical Research and the Program of the Institute: The results of this study indicate that the potentiation of $CHCl_3$ hepatotoxicity by MBK and acetone is due, at least in part, to the induction of cytochromes P-450 that metabolize $CHCl_3$ to $COCl_2$.

The major form of cytochromes P-450 induced by MBK is similar to that induced by PB. This finding suggests that these structurally dissimilar compounds cause induction by the same mechanism. Interestingly, acetone, which is structurally similar to MBK, appears to induce qualitatively different forms of cytochromes P-450.

Although it is not yet known how induction by cytochromes P-450 is controlled, the structural information obtained in this study should be useful in helping to solve this problem.

Proposed Course of Project:

The results of this study show that structurally dissimilar compounds can have similar, if not identical, induction properties. We intend to investigate further the relationship between structure and induction in order to more clearly understand how the levels of cytochromes P-450 are regulated.

Publications:

Branchflower, R.V. and Pohl, L.R.: Investigation of the mechanism of the potentiation of chloroform induced hepatotoxicity and nephrotoxicity by methyl n-butyl ketone, Toxicol. Appl. Pharmacol. 61, 407-413 (1981).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00942-02 LCP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Development of a flow cell to detect cytochromes P-450

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	R.V. Branchflower	Staff Fellow	LCP	NHLBI
Other:	L.R. Pohl	Pharmacologist	LCP	NHLBI

COOPERATING UNITS (if any)

Robert Bonner, Biomedical Engineering and Instrumentation Branch

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme-Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.35	0.35	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

During the past 10 years there has been considerable interest in isolating and characterizing the numerous species of cytochromes P-450. Separations are most often accomplished by a combination of several types of column chromatography. Progress in the field, however, has been greatly hampered by the tedious methodology for assaying cytochromes P-450. We have developed a prototype flow cell which in combination with a micro processor unit may be used to assay cytochromes P-450 as it is eluted from columns. We are able to measure and record difference spectra every 5 seconds at flow rates up to 2 ml/minute.

PHS-6040
(Rev. 2-81)

Project Description:

Objective: To develop a flow cell microprocessor unit for the continuous monitoring of cytochromes P-450 levels in eluents from either low or high pressure liquid chromatography columns.

Methods Employed: A solution of sodium dithionite (1 gm/100 ml) in a detergent buffer is degassed and kept under nitrogen. This solution is mixed via a peristaltic pump with an equal volumn of column effluent. The resulting mixture is split in half. One half goes directly to the reference flow cell and the other half flows through a CO gas exchange manifold to the sample flow cell. Measurements of 450-490 are made every 5 seconds using a Hewlett Packard Model 8450 A Spectrophotometer and plotted.

Major Findings: We are able to detect cytochromes P-450 as they elute from a DE-52 anion exchange column at flow rates of 0.5 to 2.0 ml/minute. The values obtained compare favorably with those obtained by measuring similar fractions via traditional manual methods.

Significance to Biomedical Research and the Program of the Institute: Cytochromes P-450 has been demonstrated to play an important role in drug interactions, xenobiotic toxicity, carcinogenesis, and regulation and synthesis of a number of endogenous compounds. It is suspected that different people have different compositions of cytochromes P-450. Thus the development of an analytical technique that will greatly aid us in determining the role of cytochromes P-450 in physiology and pathology.

Proposed Course of Project: The immediate course of this investigation will be to perfect the prototype system and develop a unit which can be routinely used to monitor column eluents during the analysis and purification of cytochromes P-450.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00943-02 LCP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Cultured rat hepatocytes in the study of drug-induced hepatotoxicity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Richard Chenery	Vist. Fellow	LCP	NHLBI
Other:	Mariam George	Vist. Fellow	LCP	NHLBI
	Gopal A. Krishna	Chief, Section	LCP	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Drug-Tissue Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have previously reported that cultured hepatocytes metabolize acetaminophen to glucuronide, sulfate and glutathione conjugates, even after 24-48 hours in culture. We have now characterized the relationship between acetaminophen activation and loss of plasma membrane function in these cultured cells. Correlation was observed between various parameters of plasma membrane injury e.g., leakage of cytoplasmic enzymes lactic dehydrogenase (LDH) and glutamic-pyruvic transaminase and uptake of trypan blue. Moreover we have correlated these with the morphology of the cultured hepatocytes utilizing the transmission electron microscope. The injury was found to occur in certain cells and not others: thus exhibiting as all-or-none character. The omission of $CaCl_2$ from the culture medium was found to alter the kinetics of LDH release but failed to prevent cell injury.

PHS-6040
(Rev. 2-81)

Project Description:

Objective: Acetaminophen is a commonly used analgesic that causes liver necrosis when administered in large doses. Our objective was to characterize the injury induced by acetaminophen in cultured hepatocytes; the relationship between metabolic activation and the extent of cell injury and the possible role of extracellular Ca^{2+} upon the injury process. We have previously shown that with CCl_4 extracellular Ca^{2+} stimulated injury at low concentrations of the hepatotoxin but the hydrophobic nature of the compound precluded a detailed analysis of the kinetics of the process. With acetaminophen, however, it was possible to completely separate the period required for metabolism from the subsequent changes in plasma membrane permeability.

Methods Employed: Hepatocytes from control and phenobarbital treated animals were isolated and cultured on collagen-coated petri dishes as described in our previous report. After maintenance in culture for 24 hours utilizing culture medium containing 25 mM nicotinamide the cultured cells were preincubated for a 6 hour period with 0, 1 or 6 mM acetaminophen in the presence of 3.6 mM $CaCl_2$. At the end of this preincubation period the acetaminophen was removed and replaced by fresh medium containing either no added $CaCl_2$ or 3.6 mM $CaCl_2$. Leakage of cytoplasmic enzymes, uptake of trypan blue and changes in cell morphology were then followed.

Major Findings: We have previously reported that hepatocytes maintained in culture for 24 hours retain levels of cytochrome P-450 and the ability to metabolically activate acetaminophen, the active metabolite is essentially trapped by cellular glutathione. The accumulation of the acetaminophen glutathione conjugate in these cultures is linear for six hours when acetaminophen concentrations are at or below 6 mM. Phenobarbital treatment of the rats resulted in a 2-fold increase in acetaminophen-glutathione conjugate formation. No evidence was obtained for cellular injury throughout this incubation period as judged by measurement of cytoplasmic enzyme activities in the culture medium.

The acetaminophen was then removed and replaced by fresh culture medium with or without 3.6 mM $CaCl_2$. Significant amounts of enzyme leakage occurred during the following 24 hours. LDH leakage was time and dose-dependent for hepatocytes from either control or phenobarbital treated animals.

In control cells, omission of $CaCl_2$ from the second incubation delayed the leakage of LDH but not the total quantity. With hepatocytes from phenobarbital treated rats, omission of $CaCl_2$ from the second incubation had little effect upon the early leakage of LDH, but increased the total quantity at latter times. Thus low calcium in the culture medium, markedly increased the toxicity to acetaminophen. This is in aggreement with our previous studies when we found EGTA (100μM) added to calcium free medium markedly increased acetaminophen toxicity as measured by total quantity of LDH leaked from the cells.

However, in the case of ionophore A23187 and CCl_4 we have shown that addition of $CaCl_2$ increased the observed cell injury. Thus it appears that $CaCl_2$ may influence cell injury in two divergent ways; one tends to protect the cells and the other tends to exacerbate the injury. The predominant influence appears to depend upon a number of factors; including the exact

Ca^{2+} concentration, nature of the toxicant and the pH. These observations could explain the contradictory findings of various authors reported in the literature.

A more extended incubation (24 hours) with acetaminophen resulted in LDH leakage in excess of 70%. The leakage was dose dependent and correlated well with other parameters of plasma membrane damage such as loss of GPT from the cells and increased staining by trypan blue. Studies with the transmission electron microscope indicated that at concentrations of acetaminophen at or below 0.7 mM extensive structural changes could be observed, with the exception of some swelling of the smooth endoplasmic reticulum. However, at higher concentrations of acetaminophen, cells with a discontinuous plasma membrane were observed; the contents of the cells having completely lysed. The number of such cells increased as the concentration of acetaminophen increased. At high concentrations of acetaminophen all cells appeared damaged and many lysed. Similar observations were made with the scanning electron microscopy.

Significance to Biomedical Research and the Program of the Institute: Overdoses of acetaminophen have been associated with hepatotoxicity and nephrotoxicity in humans. This study helps to define the nature of the injury induced by acetaminophen and the role of extracellular Ca^{2+} in this injury.

Proposed Course of Project: The identification and isolation of subpopulations of cells from the hepatocyte preparation remains a major goal in the study of drug-induced toxicity. The isolation of hepatocyte subpopulations with differential sensitivities to acetaminophen is presently under investigation.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00944-01 LCP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Mechanisms of drug-induced toxicity in cultured cardiomyocytes.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Mariam George	Visiting Fellow	LCP	NHLBI
Other:	Richard Chenery	Visiting Fellow	LCP	NHLBI
	Gopal A. Krishna	Chief, Section	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Drug-Tissue Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The effects of adriamycin analogs on cardiotoxicity was studied in cultured cardiomyocytes. The earliest structural effects observed in the cardiomyocytes were vacuolization and nuclear fragmentation at 4 hrs. after treatment with adriamycin (ADR) (100 ug/ml). While daunomycin (DAUN) analog of adriamycin, containing a methyl group in place of hydroxymethyl group showed much more drastic effects at lower concentration, DAUN caused an immediate cessation of beating of cells along with changes in the cell shape as well as lifting of cells from the plate. Cytoplasmic enzyme leakage into the medium was used as an index of cell toxicity induced by ADR and DAUN. Quantitative measurement of LDH leakage indicated that DAUN was 3-4 times more toxic in causing cell death than adriamycin. Measurement of malanaldehyde release indicated that adriamycin toxicity may not be related to lipid peroxidation.

PHS-6040
(Rev. 2-81)

Project Description:

Objective: Since the introduction of adriamycin as an effective anti-tumour agent, a number of reports have appeared that it induces dose dependent cardiotoxicity. Even though a number of mechanisms have been postulated, the exact mechanism by which adriamycin induces specific cardiac tissue damage remains unclear. One of the main objectives of this study has been to investigate cardiac toxicity induced by adriamycin and other analogs in a tissue culture system where various parameters such as dose, time of exposure, as well as ionic environment can be controlled to a greater extent than is possible in vivo. Previously we have utilized successfully long term cell culture systems to study the mechanism of drug-induced liver damage and the specific role of calcium ions in the drug induced cell death. We therefore propose to study the mechanism of adriamycin cardiotoxicity in rat cardiomyocyte culture.

Methods Employed: Cardiomyocytes from 4 days old neonatal rat heart were isolated by collagenase treatment. The hearts were chopped and were incubated for 1 hr. with Joklik medium containing 0.2% BSA and 0.2% collagenase. The cells that were released into the medium by enzyme treatment were collected and separated by centrifugation. The cells were resuspended in Williams Medium E containing 10^{-6}M epinepherine, 0.021 U/ml insulin, penicillin (100 units/ml), streptomycin (100 ug-eg/ml) 10% (v/v) horse serum and plated in 25 cm collagen coated Falcon bottles. The dead cells which remain unattached after 24 hrs. incubation were removed and fresh medium were added to the flask. The cells attached to the flask continued to grow reaching confluency in about 4-5 days to produce myocytes which beat synchronously. The drugs were dissolved in distilled water and added in the medium. (For treatment Earle's Balanced Salt Solution was used). Cytoplasmic enzyme leakage was measured. For electron microscopic studies the cells in the flasks were dehydrated and embedded while attached to the flask. The cells both control and treated were fixed with 2% paraformaldehyde gluteraldehyde in 0.15 M sodium cacodylate buffer for 20 min., washed with the buffer and post fixed with 2% osmium tetroxide in H_2O for 20 min., then dehydrated with ethanol and embedded with Epon 812. Serial sections of myocardial cells were stained with uranyl acetate and lead citrate. These sections were examined under the electron microscope.

Major Findings: After 7 days in culture, the cells became confluent by the growth of muscle cells. At this time the cells beat rhythmically and synchronously. Changing the medium with 10^{-6}M epinepherine for every 48 hours helps to maintain cell beating. The earliest structural effects observed in the myocytes were vacuolization and nuclear fragmentation at 4 hrs after treatment with adriamycin (100 ug/ml). While daunomycin, an analog of adriamycin, containing a methyl group in place of hydroxymethyl group showed much more drastic effect at lower concentrations. Daunomycin caused an immediate cessation of beating of cells along with marked changes in cell shape as well as lifting of cells from the plate. Quantitative measurement of LDH leakage indicated that daunomycin was 3-4 times more toxic in causing cell death than adriamycin. Moreover, daunomycin induced cardiac cell arrest at lower concentrations than adriamycin. Measurement of malanaldehyde release indicated that adriamycin toxicity may not be related to lipid peroxidation. Preliminary results from electron microscopic studies indicate that the confluent cells contain array of sacroplasmic tubules

arranged in bundles and the toxicity induced by adriamycin manifested as vacuoles in the sarcoplasmic reticulum. The toxicity induced by adriamycin thus appears to be similar as seen in vivo using adult animals. However, more studies are required to confirm these findings.

Significance to Biomedical Research and the Program of the Institute: The finding that the toxicity of adriamycin with cultured cells is similar to that found in vivo should greatly aid in the understanding of the mechanism of cardiac toxicity induced by drugs. The finding that daunomycin which is an analog of adriamycin containing a methyl group instead of hydroxymethyl group is more toxic than adriamycin in causing cell death indicates that a small minor modification in chemical structure can markedly reduce cardiac toxicity without changing the anti-tumour activity of the drug.

Proposed Course of the Project: We propose to study the cardiac toxicity of various analogs of adriamycin in order to understand structural requirement for the cardiac toxicity of adriamycin and daunomycin. It may be possible to modify these drugs by simple chemical treatment which should greatly alter the toxic potential without altering their therapeutic efficacy.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00945-01 LCP

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Polymorphic hydroxylation of desipramine in man.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Elizabeth A. Lane	Visiting Fellow	LCP	NHLBI
Other:	James R. Gillette	Chief	LCP	NHLBI

COOPERATING UNITS (if any)
Dr. W.Z. Potter, Clinical Psychobiology Branch, NIMH
Dr. M.V. Rudorfer, Clinical Psychobiology Branch, NIMH

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme-Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Fourteen Chinese and sixteen Caucasian normal volunteers were administered a single dose of desipramine. Plasma and urine samples were analysed for DMI and an active metabolite, hydroxydesipramine. To date it appears that the proportion of Chinese that are slow hydroxylators of desipramine is larger than for Caucasians. This may be the reason for tricylic antidepressant dosing of Chinese at lower rates compared with Caucasians. In addition, the renal clearance of hydroxydesipramine was lower in older subjects.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: There have been several reports of a tendency for lower doses of tricylic antidepressants (TCAs) to be used in treatment of Chinese compared to Caucasian patients. Hydroxylation is regarded as a major pathway of TCA metabolism and the distribution of the ability to hydroxylate a number of drugs has been shown to be multimodal. Furthermore, the proportion of different ethnic populations being slow hydroxylators has been shown to vary. Therefore, the hydroxylation of desipramine was investigated in Chinese and Caucasian normal volunteers.

Methods Employed: Established methods were adapted to this study.

Major Findings: Although the study is still in an early stage, the hydroxylation clearance of desipramine appears to be smaller in Chinese than in Caucasians. This investigation has provided the means of studying other variables that govern the concentration of desipramine metabolites. In particular, the renal clearance of hydroxydesipramine appears to decrease with the age of the subject.

Significance to Biomedical Research and the Program of the Institute: (1) Pharmacogenetics is a variable involved in drug disposition and may contribute significantly to the wide range of doses of some drugs required in therapy. (2) Many drugs are hydroxylated to active or inactive metabolites. A better understanding of how significantly a particular clearance contributes to the determination of metabolite concentration provides an improved basis for dosing modification in compromised patients. (3) As the TCAs are tested for antiarrhythmic activity, an understanding of the determinants of concentrations of drug and active metabolites may facilitate this evaluation.

Proposed Course of Project: Debrisoquine has been used to phenotype slow and rapid hydroxylators. Identified slow and rapid hydroxylators of desipramine will be tested with a single dose of debrisoquine in order to determine whether the two phenotypes are the same.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00946-01 LCP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Mechanism of differences in metabolism and toxicity of chloroform and bromoform

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Patricia Morris	Chemist	LCP	NHLBI
Other:	Lance R. Pohl	Pharmacologist	LCP	NHLBI
	John W. George	Chemist	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme-Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.80	0.10	0.70

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The administration of CHCl3 to phenobarbital pretreated rats produced a 65 percent decrease in liver glutathione (GSH). In contrast, CHBr3 treatment led to only 14 percent depletion of GSH. CHCl3 was activated to phosgene (COCl2) by rat liver microsomes, which reacted with glutathione (GSH) to produce predominantly diglutathionyl-dithiocarbonate (GSCOSG). CHBr3, however, was converted by microsomes into carbonyl dibromide (COBr2) which reacted with GSH to produce oxidized glutathione (GSSG) and very little GSCOSG. These results indicate that the hepatotoxic metabolites of CHCl3 and CHBr3, namely COCl2 and COBr2 respectively, react differently with tissue components and therefore may cause tissue necrosis by different mechanisms. By understanding the basis of these differences, a better fundamental knowledge of the events leading to tissue necrosis should be obtained.

PHS-6040
(Rev. 2-81)

Project Description:

Objective: To determine whether the hepatotoxic metabolites of $CHCl_3$ and $CHBr_3$, namely phosgene ($COCl_2$) and carbonyl bromide ($COBr_2$) react similarly with endogenous molecules.

Methods Employed: Rats were administered $CHCl_3$ or $CHBr_3$ (3.73 mmol/kg in sesame oil, intraperitoneally) and after 1 and 4 hours liver glutathione (GSH) levels were measured by published methods. The metabolism of $CHCl_3$ and $CHBr_3$ by rat liver microsomes in the presence of GSH was compared by measuring the metabolites by HPLC. The reaction of $COCl_2$ or $COBr_2$ with GSH in aqueous media was also analyzed by HPLC.

Major Findings: One hour after the treatment of phenobarbital pretreated rats with $CHCl_3$, the level of liver GSH was decreased by 65 percent from normal values. After 4 hours, the level of GSH was depressed by 68 percent. In contrast, one hour after treatment of rats with $CHBr_3$ the level of liver GSH decreased by only 14 percent. After 4 hours, the amount of liver GSH had returned to normal levels.

Approximately 180 nmol/mg protein/15 min of $CHCl_3$ was metabolized to GSCOSG by rat liver microsomes in the presence of GSH, whereas only 17 nmol/mg protein/15 min of $CHBr_3$ was metabolized to GSCOSG.

When 0.5 umoles of $COCl_2$ was allowed to react with 5 umol of GSH at pH 7.5, more than 69 percent of the $COCl_2$ was converted to GSCOSG. In contrast, only 7 percent of $COBr_2$ was transformed to GSCOSG. More than 74 percent of $COBr_2$, however, was converted into oxidized glutathione (GSSG). No GSSG was detected as a product in $COCl_2$ reaction mixture.

Significance to Biomedical Research and Program of the Institute: We have previously reported that $CHCl_3$ and $CHBr_3$ are approximately equipotent as hepatoxins and their toxicity is dependent upon their metabolism into $COCl_2$ and $COBr_2$, respectively. It has now been shown, however, that these structurally similar metabolites react differently with endogenous molecules, such as GSH. For example, phosgene ($COCl_2$) reacts predominantly with GSH to produce GSCOSG; this reaction explains how $CHCl_3$ depletes liver GSH. In contrast, $COBr_2$ reacts with GSH to produce GSSG. Rapid reduction of GSSG by liver GSH reductase probably explains why very little GSH is depleted after the administration of $CHBr_3$ to rats.

These results are important to the field of chemical toxicity for the following reasons: 1) They emphasize that not all toxic reactive electrophilic metabolites deplete liver GSH. 2) Depletion of liver GSH to a particular threshold is not always a prerequisite for a reactive metabolite to produce tissue damage. In some cases, the metabolite may react so rapidly with vital tissue components that its scavenging by GSH is not an important detoxification reaction. This appears to be the case with $COBr_2$ but not $COCl_2$. 3) Alternatively however, $COBr_2$ might react with GSH to produce short-lived, localized zones of GSSG and GSH depletion, where the reactive metabolite can react more selectively with vital tissue components. During the preparation of the tissue samples for GSH determination, the GSSG might become reduced to GSH, so that

no net loss of GSH is observed. If this mechanism were tenable, it might explain what occurs in the liver after the administration of other toxins that lead to momentary increases in tissue GSSG, such as possibly carbon tetrachloride. 4) If $COCl_2$ and $COBr_2$ react differently with other tissue components, they may cause tissue necrosis by different pathways. By studying these differences in detail, a better understanding of how reactive metabolites cause tissue damage in general should be obtained.

Proposed Course of Project: We plan to determine the basis of the differences of the chemistry of $COCl_2$ and $COBr_2$ in order to better understand more precisely how $CHCl_3$ and $CHBr_3$ produce liver necrosis. Our initial goal will be to elucidate the mechanisms of the reactions of $COCl_2$ and $COBr_2$ with GSH.

Publications:

Pohl, L.R., Branchflower, R.V., Highet, R.J., Martin, J.L., Nunn, D.S., Monks, T.J., George, J.W., and Hinson, J.A.: The formation of diglutathionyl dithiocarbonate as a metabolite of chloroform, bromotrichloromethane, and carbon tetrachloride, Drug Metab. Disp. 9:334-339 (1981).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00947-01 LCP

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Formation of a glutathione conjugate of propranolol metabolism by rat liver enzymes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Henry A. Sasame	Chemist	LCP	NHLBI
Other:	None			

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Enzyme-Drug Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Rat liver microsomes convert propranolol to three major metabolites; 5-hydroxypropranolol (5OHPr), 4-hydroxypropranolol (4OHPr) and desisopropyl-propranolol (Desiprpr). In the presence of both hepatic soluble enzymes and reduced glutathione, the formation of 5-hydroxypropranol was markedly diminished with concommitant increase of glutathione conjugate. More glutathione conjugate was formed with liver microsomes isolated from pretreated with BNF than with those from phenobarbital treated or untreated rats. Accordingly, the covalent binding of active intermediate from propranolol metabolism to a macromolecules of rat liver microsomes was highest with liver microsomes isolated from BNF treated rats.

355

Project Description:

Objective: Recently, Schneck's group at Hershey Medical College, Pa. showed that liver microsomes isolated from rats pretreated with phenobarbital convert propranolol to a metabolic inhibitor or P-450 complex, possibly through the formation of 4-hydroxypropranolol. They have also shown that propranolol becomes covalently bound to microsomal protein. But whether the two effects are related remains to be determined. In order to evaluate their hypothesis, we have undertaken a series of experiments designed to trap the reactive metabolite as glutathione conjugate.

Methods Employed: Standard biochemical procedures have been applied, except that radiolabeled S-propranolol (^{3}H at 4 position of naphthelenl ring) has been adapted to search for propranolol GSH conjugate.

Major Findings: 1) The extent of *in vitro* covalent binding of a chemically reactive intermediate from propranolol by liver soluble fraction and microsomes isolated from untreated, phenobarbital and β-naphthoflavone treated rats was as follows: (nmol/mg/10 min); control 0.2; PB 0.28; BNF 0.36. The addition of reduced GSH decreased these amounts to 10-20% of the original.

2) The presence of reduced GSH together with liver soluble fraction and microsomes isolated from BNF treated rats enhanced preferentially the radioactivity in the remaining aqueous phase after ethyl acetate extraction of propranolol metabolite(s) at pH 9.5. The differential activity in aqueous phase between the presence and absence of GSH was as follows: (nmole/mg/microsomes/10 min) BNF 4.5; PB 2.66; control 2.75.

3) Analysis of water soluble fraction by HPLC revealed that GSH resulted in formation of a possible GSH conjugate of propranolol and a 60% decrease in formation of 5OHPr, but no change in the formation of either 4OHPr or DesiprPr.

Significance to Biomedical Research and the Program of the Institute: There is evidence that repeated administration of propranolol to patients decreases the metabolism of other drugs. The mechanism of the effect, however, remains unproven.

Proposed Course of the Project: 1) During the course of screening for cross reactivity of monoclonal antibody (antibody against cytochrome P-450 induced by 3MC treatment with rats) against various animal species, we found that liver microsomes isolated from hamster treated with BNF forms unusually large amounts of 5OHPr. Since this metabolite may be derived from active intermediate, we should determine whether propranolol causes liver damage in hamsters treated with BNF.

2) Through the use of double labeled isotope technique-C^{14}-glutathione and H^{3}-propranolol, we will be able to identify the glutathione-conjugate and determine the chemical structure through the use of high performance liquid chromatography and mass spectrometry.

3) The effects of glutathione on the formation of metabolic inhibitor complexes will be studied.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00948-01 LCP

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Use of monoclonal antibodies to study species differences in cytochromes P-450

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Henry A. Sasame	Chemist	LCP	NHLBI
Other:	James R. Gillette	Chief	LCP	NHLBI
	Richard V. Branchflower	Staff Fellow	LCP	NHLBI

COOPERATING UNITS (if any)

Dr. Harry Gelboin, Laboratory of Molecular Carcinogenesis, NCI.

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Enzyme-Drug Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.8	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The relative rates of formation of 4-hydroxy propranolol (4OH Pr), 5-hydroxy propranolol (5OH Pr) and desisopropyl propranolol (DI Pr), from the R-isomer of propranolol, a widely used β-adrenergic blocker, varies with the treatment with the animal species. A monoclonal antibody raised against cytochrome P-450 induced by 3-methyl cholanthrene inhibited the metabolism of the R isomer of propranolol by liver microsomes from rats, mice, guinea pigs and rabbits treated with β-naphthoflavone (βNF) but it did not inhibit propranolol metabolism by liver microsomes from hamsters, treated with βNF. Since the relative amounts of 4OH Pr, 5OH Pr and DI Pr inhibited by the antibody differed with the animal species, it seems likely that the antibody inhibits different forms of cytochrome P-450.

PHS-6040
(Rev. 2-81)

Project Description:

Objective: Last year we presented evidence for regioselectivity in the metabolism of propranolol by rat liver microsomes which depended upon the type of pretreatment of the rats. Availability of monoclonal antibody against cytochrome P-450$_c$ (a major form of P-450 inducable by 3MC in rats), developed by Dr. Harry Gelboin at NCI, provides a way of studying species (and tissue) differences in the cytochromes P-450 which catalyze the formation of propranolol metabolites.

Methods Employed: The experimental techniques were essentially the same as those used in the study of propranolol metabolism except that each metabolite was assayed in a high performance liquid chromatograph equipped with a fluorescence detector (FS970 L.C. Fluorometer, Schoeffel Instrument Corp.).

Major Findings: 1) Rats - The monoclonal antibody inhibited the conversion of R-propranolol to 4-hydroxy propranolol (4OH Pr), 5-hydroxy propranolol (5OH Pr) and desisopropyl propranolol (DI Pr) by liver microsomes from β-naphthoflavone (βNF) treated rats by 81, 84, and 75% respectively. Thus the form of cytochrome P-450 inhibited by the antibody accounts for most of the propranolol metabolism by these liver microsomes. Because the Km values for the inhibitable reactions were nearly identical, we could obtain no evidence that the antibody reacts with several forms of cytochrome P-450 having different affinities for propranolol. Moreover, 4OH Pr/5OH Pr ratio and the DI Pr/5OH Pr ratio of the inhibited activities (1.22 and 6.92 respectively) were similar to those of the residual activities (1.29 and 12.8 respectively). Thus there was no convincing evidence that the residual activity for 4OH Pr formation was due to a different form of cytochrome P-450, though the evidence suggests that the residual activity for DI Pr may have been due in part to another form of cytochrome P-450.

2) Mice - The monoclonal antibody inhibited the formation of 4OH Pr by 35% and the formation of 5OH Pr and DI Pr by 70% by liver microsomes from mice treated with βNF. Accordingly the 4OH Pr/5OH Pr ratio for residual activity was 2.45 whereas that of the inhibitable activities was 0.58. Thus the microsomes must have contained more than one form of cytochrome P-450 that metabolizes R-propranolol. Of the activities inhibited by the antibody, the 4OH Pr/5OH Pr ratio (0.58) and DI Pr/5OH Pr ratio (18.7) obtained with the mouse liver microsomes differed from those obtained with rats. This suggests that the form of cytochrome P-450 in mouse liver microsomes that combines with the antibody is functionally different from the form of cytochrome P-450 in rat liver microsomes that combines with the antibody.

3) Guinea Pigs - With liver microsomes from guinea pigs treated with βNF, the antibody inhibited the formation of 5OH Pr by 87%, 4OH Pr by 80% and DI Pr by only 20%. Thus the microsomes must have contained at least

two forms of cytochrome P-450 that catalyze the metabolism R-propranolol. Of the activities inhibited by the monoclonal antibody, the 4OH Pr/5OH Pr ratio (0.28) and the DI Pr/5OH Pr ratio (0.95) differed from those obtained with liver microsomes from either rats or mice treated with βNF. This suggests that form of cytochrome P-450 in guinea pig microsomal inhibited by the antibody differed from that present in either rats or mice.

4) Rabbit - With liver microsomes from rabbits treated with either βNF or 3-methylcholanthrene, the antibody inhibited the formation of DI Pr by over 90%, but did not inhibit the formation of either 4OH Pr or 5OH Pr. Thus the hydroxylated metabolites are formed by a different form of cytochrome P-450 than is DI Pr in rabbit liver microsomes. Moreover the form of cytochrome P-450 inhibited by the antibody in rabbit microsomes is functionally different from the forms of cytochrome P-450 inhibited by the antibody in liver microsomes from rats, mice or guinea pigs.

5) Hamsters - Although the metabolism of R-propranolol by liver microsomes from hamsters treated with βNF was greater than that by microsomes from rats treated with βNF, the antibody did not inhibit the formation of any of the metabolites by hamster liver microsomes. Thus the forms of cytochrome P-450 induced by βNF in hamsters clearly differ from those induced by βNF in the other animal species.

6) The antibody inhibited the metabolism of benzpyrene by liver microsomes from different species of animals treated with βNF to different extents: rats, 94%; mice, 60%; guinea pig, 23%; hamster, 33%; and rabbit, 60%. Thus benzpyrene may be metabolized by more than one form of cytochrome P-450 in liver microsomes from mice, guinea pig, hamster and rabbits. Indeed the formation of one of the R-propranolol metabolites may be a more specific assay of the inhibitable cytochrome P-450 in liver of these animal species than is the hydroxylation of benzpyrene. Moreover, the antibody does not cross-react with purified LM-4, isolated from rabbit liver; thus the form of cytochrome P-450 in rabbit liver microsomes inhibited by the antibody cannot be LM-4.

Significance to Biomedical Research and the Program of the Institute: This study clearly shows that a monoclonal antibody can inhibit more than one form of cytochrome P-450. The approach used in this study should be useful in establishing the substrate specificity of enzymes that metabolize propranolol in various species.

Proposed Course of Project: The further kinetic analysis of propranolol metabolism from propranolol should be carried out with the purified cytochrome enzyme isolated from liver microsomes from various inducers along with monoclonal antibody against each specific inducer.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00949-01 LCP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Effect of Thyroid Status on Acetaminophen Metabolism in Cultured Hepatocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Richard Chenery	Vist. Fellow	LCP	NHLBI
Other:	Gopal A. Krishna	Chief, Section	LCP	NHLBI
	Sue Lyons	Staff Fellow	LBM	NIADD

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Drug Tissue Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have prepared isolated hepatocytes from thyroidectomized and sham operated adult rats. There was a decrease in hepatocyte size and protein content per cell in the thyroidectomized animals. When the metabolism of acetaminophen is expressed per 10^6 cells thyroidectomy resulted in an increase in glucuronidation, and decreases in sulfation and glutathione conjugate formation. When the metabolism is expressed per mg of cell protein, glucuronidation was greatly increased in the thyroidectomized animal, sulfation remained little changed and glutathione conjugate formation was decreased. The decrease of glutathione conjugate formation was also observed in the 9000 x g supernatant of liver to which cofactors were added, indicating a decrease in the cytochrome P-450 specie(s) responsible for its activation. Levels of total cytochrome P-450 were not decreased when expressed per mg of protein. However, increases in phenolsulfotransferase activity were noted in the soluble fraction, so that it appeared that the activity of the transferase was not the limiting factor in acetaminophen sulfation. Administration of thyroid hormones either in vivo or in tissue culture failed to alter either glucuronidation or sulfation, but had complex effects upon glutathione conjugate formation.

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PHS-6040
(Rev. 2-81)

Project Description:

Objectives: Factors controlling drug-metabolism in cultured hepatocytes are numerous and difficult to identify. However, the numerous effects of thyroid hormones upon drug metabolism indicated that an investigation of the effects of thyroid status in vitro may represent a rational approach to the study of control mechanisms of drug metabolism in cultured cells.

Methods Employed: Isolated hepatocytes were prepared and cultured as described previously. The measurement of cytochrome P-450 and acetaminophen conjugates were measured by established methods. The effects of triiodothyronine and thyroxine were investigated both in vivo and in vitro. For the studies of the role of sulfate availability we utilized both Williams Medium E and 10 mM HEPES buffered saline, free from added amino acids or inorganic sulfate (^{35}S) sodium sulfate and ^{35}L cysteine were utilized as radiolabel. For the studies of acetaminophen glutathione conjugate formation we incubated post mitochondrial liver supernatants (PMS) with (^{3}H) acetaminophen at pH 7.4, along with 4 mM glutathione and 1 mM NADPH.

Major Findings: Within 14 days of thyroidectomy, male rats fail to demonstrate an increase in total body weight. However, isolated hepatocytes may be prepared in high yield from these animals and introduced into maintenance culture. The hepatocytes are smaller and contain less total protein per cell compared to cells from sham operated rats. The metabolism of acetaminophen to glucuronide, sulfate and glutathione conjugates was measured in sham, thyroidectomized, T_4-injected thyroidectomized and cultured hepatocytes.

a) Glucuronide formation: Glucuronide formation was elevated in thyroidectomized hepatocytes when the data are expressed either per 10^6 cells or per mg cell protein. Injection of T_4 to thyroidectomized animals or addition of T_3 to cultured cells failed to induce any detectable change in glucuronidation.

b) Sulfation: Acetaminophen sulfate formation was greatly decreased per 10^6 cells in thyroidectomized rats, but the differences were much smaller when expressed per mg of protein. Acetaminophen sulfate formation was nearly abolished in medium in which inorganic $SO_4^=$ and sulfur amino acids were omitted. Addition of $MgSO_4$ stimulated acetaminophen sulfate production in cells from both sham and thyroidectomized animals, the difference in rate of acetaminophen sulfate formation was more apparent at 5 mM $MgSO_4$ than 0.1 mM $MgSO_4$. However, the rate of acetaminophen sulfate formation supported by 4 mM L-cysteine was not statistically different in hepatocytes from either sham or thyroidectomized rats. These results are consistent with the idea that availability of sulfate rather than the activity of sulfotransferases may determine the rate of acetaminophen sulfate formation. The uptake of inorganic sulfate into hepatocytes is a saturable process with an apparent K_m (0.5 mM) very close the the apparent K_m of acetaminophen

sulfate formation, with respect to sulfate concentration.

The activity of sulfotransferase was measured at both pH 7.4 and pH 5.5, utilizing 2 mM acetaminophen as substrate. The activity was slightly reduced on a wet weight basis but elevated on a protein basis, in thyroidectomized animals, compared to sham operated animals.

Glutathione Conjugate Formation: Glutathione conjugate was formed in reduced amounts in hepatocytes from thyroidectomized rats, regardless of the method of expressing the data. This decrease is also seen in post mitochondrial fractions (9000 x g) to which cofactors were added. Various treatments altered the activity of conjugate formation in intact cells expressed as pmol/hr/pmol of cytochrome P-450, over a 4-fold range, indicating that specific molecular forms of the cytochrome may be involved in the metabolism of acetaminophen.

Further evidence that specific cytochrome P-450's might be involved comes from Lineweaver-Burke plots of glutathione conjugate formation with respect to acetaminophen concentration. In control and sham cells the plot is biphasic (K_m, 1.50 $\pm$ 0.25 (n=5) and 0.30 $\pm$ 0.10 (n=4) mM). In a variety of experimental and culture situations, however, only a single straight line was detectable. In phenobarbital-induced and cultured-thyroidectomized hepatocytes only the high K_m activity was observed, whereas in T_4-injected-thyroidectomized animals only the low K_m form was detected.

At high concentrations thyroid hormones decreased total cytochrome P-450 levels both *in vivo* and *in vitro*.

Significance to Biomedical Research and to the Program of the Institute: Acetaminophen is a commonly used analgesic, severe overdose of which has been closely associated with hepatotoxicity and neprotoxicity in humans. Thyroid disfunction is relatively common an our findings indicate that complex interactions may occur in individuals with unusual thyroid status. Altered thyroid status changes the T-1/2 of acetaminophen in human subjects. However, our experiments demonstrate the potential complexity of the changes involved and confirm the importance of thyroid status in drug metabolism.

Proposed Course of Project: We propose to investigate the role of thyroid status and sulfate availability in other drug transformation and toxicity.

Liver cells from hypothyroid rats exhibited reduced sulfate conjugating ability, but levels of sulfotransferases were not reduced. We have recently shown that uptake of sulfate can be rate-limiting, and that $MgSO_4$, L-cysteine and methionine act as sulfate donors. We are presently investigating if the uptake and availability of sulfate for PAPS synthesis in hypothyroid cells may be the key factor in the reduction of sulfate conjugation in cells.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00950-01 LCP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Diffusion of reactive metabolites out of hepatocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Serrine S. Lau	Vist. Fellow	LCP	NHLBI
OTHERS:	Terrence J. Monks	Vist. Assoc.	LCP	NHLBI
	James R. Gillette	Chief	LCP	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.75	0.75	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Reactive metabolites of bromobenzene are capable of leaving the intact hepatocyte and becoming covalently bound to externally added protein. Varying the amounts of externally added protein increases the amount of covalently trapped material, enabling an estimation of the fraction of the compound which can escape the liver and reach extrahepatic binding sites. As much as 35% of the reactive bromobenzene-3,4-oxide is capable of escaping the intact hepatocyte.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: One of the problems in studying the toxicity of chemically reactive metabolites is determining whether or not such metabolites are capable of leaving the cell in which they are formed and of mediating toxicities in other tissues. Thus, using bromobenzene as a model compound we were interested in developing a technique which would enable us to determine the relative amounts of reactive metabolites that leave hepatocytes.

Methods Employed: Isolated hepatocytes were prepared by collagenase perfusion as described previously by this laboratory. Isolated hepatocyte suspensions are incubated with 3 mM ^{14}C-bromobenzene in the presence and absence of various amounts of externally added nucleophile (bovine serum albumin [BSA] or glutathione-S-transferase B [GSH-TB]). After incubation the exogenous protein is separated from the hepatocytes by centrifugation. The supernatant is then subjected to sepacryl S-200 gel filtration column chromatography. The exogenous protein is completely separated from other constituents in the supernatant and the amount of radiolabel associated with the reisolated protein is determined. Furthermore, metabolites of bromobenzene, namely by the glutathione conjugates, the dihydrodiol, bromocatechol, o- and p-bromophenol, o- and p-bromophenol glucuronide were guantitated by an HPLC assay.

Major Findings: More radioactivity was trapped by 1.8 mg/ml GSH-TB (4.72 nmole/ml 30 min) than by 2.0 mg/ml BSA (1.60 nmoles/ml/30 min) which probably reflects the former's known properties as the major ligand binding protein, ligandin. Moreover, varying the amount of externally added protein increases the amount of covalently trapped material. Analysis of the data indicated that as much as 35% of bromobenzene-3,4-oxide is capable of escaping the intact hepatocyte. Moreover, GSH-TB decreased the formation of dihydrodiol, o- and p-bromophenol, bromocatechol, and o- and p-bromophenol glucuronide, but did not affect the formation of bromobenzene GSH-conjugates and intracellular covalent binding. Kinetic analysis of the data showed that all of the bromobenzene GSH-conjugates were formed within hepatocytes while rearrangement of bromobenzene-3,4-oxide to p-bromophenol occurred exclusively outside the hepatocytes.

Significance to Biomedical Research and to the Program of the Institute: The results indicate that reactive metabolites of bromobenzene are indeed capable of leaving the intact hepatocyte and of therefore mediating extrahepatic toxicity. Moreover, the differences in the ability to trap reactive metabolites by different proteins illustrates the fact that certain "target" proteins may have a higher affinity for reactive metabolites than other proteins. This selective binding may play a role in either the toxicity mediated by, or the protection from, chemically reactive metabolites. The consequences will be dependent upon whether the covalent binding leads to modificiation and inactivation of the protein concerned.

Proposed Course of Project: We intend to use the present technique to determine the ability of reactive metabolites of other toxic compounds to leave the hepatocyte.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00951-01 LCP

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Mechanism of extrahepatic bromobenzene toxicity and covalent binding

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Serrine S. Lau	Vist. Fellow	LCP	NHLBI
OTHERS:	Terrence J. Monks	Vist. Assoc.	LCP	NHLBI
	James R. Gillette	Chief	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Enzyme Drug Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.75	0.75	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Both lung and kidney microsomes possess the necessary enzymes to convert bromobenzene to p-bromophenol and p-bromophenol to bromocatechol and to covalently bound material. Moreover, a portion of the extrahepatic covalent binding derived from bromobenzene can be attributed to reactive metabolites of p-bromophenol which presumably are formed from the oxidation of bromocatechol to the quinone. The role of these reactive bromophenol metabolites in bromobenzene induced toxicity is unclear.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: The source and nature of the extrahepatic covalently bound material following bromobenzene administration is unclear. It was suggested that bromobenzene 3,4-epoxide generated in the liver is suficiently stable to leave the liver and reach extrahepatic target sites. However, we have recently shown that a major bromobenzene metabolite, p-bromophenol gives rise to nontoxic reactive metabolites that covalently bind to liver, lung and kidney tissue *in vivo*. The present study was, therefore, undertaken to determine whether the necessary enzyme system(s) responsible for the activation of bromobenzene and p-bromophenol are present in extrahepatic tissue, and to ascertin the nature of the resulting reactive metabolites.

Methods Employed: The metabolism and covalent binding of ^{14}C-bromobenzene and ^{14}C-bromophenol was investigated in rat liver, lung, and kidney microsomes. A major metabolite of p-bromophenol was identified as bromocatechol and an HPLC assay was developed to quantitate this metabolite Inasmuch as superoxide anion can oxidize catechols to quinones, which may covalently bind to protein, the contribution which bromocatechol may make to covalent binding and toxicity was investigated.

Major Findings: Both lung and kidney microsomes possess the necessary enzymes to convert bromobenzene to bromophenol and bromophenol to both bromocatechol and covalently bound material. Epoxide hydrolase inhibits the covalent binding of bromophenol implicating the formation of a phenol-epoxide. Superoxide dismutase, ascorbic acid and catechol-O-methyl transferase inhibit covalent binding of bromophenol metabolites probably generated from the oxidation of bromocatechol to microsomal protein.

Administration of either bromobenzene or bromocatechol to mice at doses of 1.9 and 2.6 mmol/kg gave rise to comparable elevations in SGPT levels, whereas at doses of 0.6 and 1.3 mmol/kg bromocatechol gave higher SGPT values than bromobenzene. Bromocatechol can be formed either via dehydrogenation of the dihydrodiol of bromobenzene or from oxidation of bromophenol. Since we have demonstrated that bromocatechol but not bromophenol causes toxicity *in vivo*, it may be important to determine whehter bromocatechol is predominantly formed via the dihydrodiol or p-bromophenol pathway.

Significance to Biomedical Research and to the Program of the Institute: The liver is the major site of toxication and detoxication of xenobiotics. However, toxicity can also be mediated in extrahepatic tissues. The results show that both lung and kidney tissue posses the necessary enzymes to convert bromobenzene to chemically reactive metabolites. Moreover, the extrahepatic toxicity mediated by bromobenzene *in vivo* may be due to metabolites other than epoxide intermediates. The participitation of more than one reactive metabolite in chemically induced toxicity illustrates the complexity in extrapolating *in vitro* studies to toxicity *in vivo*.

Proposed Course of Project: We intend to better define the contribution of bromocatechol to the *in vivo* toxicity mediated after bromobenzene administration to rats.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00952-01 LCP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

The role of p-bromophenol in bromobenzene-induced toxicity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Terrence J. Monks	Vist. Assoc.	LCP	NHLBI
Other:	James R. Gillette	Chief	LCP	NHLBI

COOPERATING UNITS (if any)

Jack A. Hinson, Carcinogenesis Division, National Center for Toxicologicl Research, Jefferson, Ark. 82079

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme-Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.25	0.25	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A hepatotoxic dose of bromobenzene (3 mmoles/kg) decreases hepatic glutathione concentration in rats by approximately 80% within 5 hr following i.p. injection. A major bromobenzene metabolite, p-bromophenol at a similar dose did not significantly alter hepatic glutathione levels. Twenty four hours after administration, serum glutamate pyruvate transaminase (SGPT) levels were significantly increased by bromobenzene but not by p-bromophenol. After 14C-bromobenzene administration, a significant amount of covalently bound radiolabel was detected in liver, kidney, small intestine and lung. After a similar dose of 14C-bromophenol, covalently bound material was found in liver (62% of the amount detected with 14C-bromobenzene) and to a smaller extent in kidney, small intestine and lung. Thus the hepatotoxicity and glutathione depleting ability of bromobenzene are mediated mainly by bromobenzene 3,4-oxide rather than by chemically reactive metabolites of p-bromophenol derived from bromobenzene. Covalently bound radiolabel from 14C-bromobenzene, however, may be derived from both bromobenzene 3,4-oxide and the nontoxic reactive metabolites of p-bromophenol.

369

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: After the administration of radiolabeled bromobenzene covalently bound label is found not only in the liver but also in several other tissues and blood plasma. The nature and source of this covalently bound material is a matter of conjecture. Although various studies have suggested that the toxic metabolite is bromobenzene 3,4-oxide they do not exclude the possibility that the toxicity might be caused by a metabolite derived from it. For example, treatments which alter the covalent binding and toxicity of bromobenzene also cause corresponding alterations in the amounts of p-bromophenol formed. Thus, the recent finding that p-bromophenol may be converted to chemically reactive metabolites by rat liver microsomes raised the possibility that p-bromophenol or one of its metabolites might cause the hepatic necrosis attributed to bromobenzene. Since neither the toxicity not the covalent binding of bromophenol have been studied in vivo the present investigation was carried out to test these possibilities.

Methods Employed: Both a time and dose response curve was determined for both bromobenzene and p-bromophenol mediated glutathione depletion. Similarly the toxicity of both compounds was determined by SGPT activities, 24 hours after dosing with varying concentrations of either compounds. Histological examination of liver and kidney sections correlated with the severity of hepatic necrosis as determined by changes in SGPT activities. The covalent binding of ^{14}C-bromobenzene and ^{14}C-p-bromophenol to rat liver, lung, small intestine and kidney protein in vivo, was determined by methods previously described by this laboratory.

Major Findings: Following the i.p. injection of bromobenzene to rats there was a dose related decrease in liver glutathione levels, as has been previously reported. However, rats given i.p. injection of bromophenol at equivalent doses showed statistically insignificant decreases in liver glutathione levels.

Bromobenzene caused a significant elevation of SGPT levels at doses of 1.8 mmole/kg and above. Histological examination of the livers of the animals revealed that their high SGPT values were probably due to centrilobular hepatic necrosis. In contrast, p-bromophenol was found to be nontoxic to rats determined by the SGPT assay. Histological examination of the livers of these animals also showed no significant pathological alterations.

After ^{14}C-bromobenzene a considerable amount of covalently bound radiolabel was detected in the target tissue, liver, as well as in the nontarget tissues kidney and small intestine. A small amount of covalently bound radiolabel was also detected in the lung. At the same dose significant amounts of covalently bound radiolabel was detected in liver after ^{14}C-p-bromophenol administration whereas smaller amounts were detected in kidney, small intestine and lung.

Significance to Biomedical Research and the Program of the Institute: These results are consistent with the view that the hepatotoxicity and glutathione depleting ability of bromobenzene are mediated by bromobenzene 3,4-oxide rather than the chemically reactive metabolites of p-bromophenol.

However, the covalent binding studies indicate that a portion of the covalent binding might be mediated by p-bromophenol since this metabolite was significantly bound to liver protein and to a lesser extent to protein in kidney, small intestine and lung.

The present data therefore emphasizes the view that estimating the total amount of covalently bound metabolites in a tissue cannot be used to predict the toxicity of a compound. The finding of covalently bound material in lung and kidney tissues after both bromobenzene and p-bromophenol administration raises the possibility that either reactive metabolites from both compounds are generated in the liver and transported to extrahepatic target sites or that p-bromophenol generated from bromobenzene may be transported and activated by both lung and kidney tissue.

Proposed Course of Study: We plan to investigate whether or not the necessary enzyme systems responsible for the activation of both bromobenzene and p-bromophenol are present in extrahepatic tissues. This is presented in the following project report.

Publication:

Monks, T.J., Hinson , J.A., and Gillette, J.R.: Bromobenzene and p-bromophenol toxicity and covalent binding *in vivo*. Life Sciences 30: 841-848 (1982).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00953-01 LCP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Mechanism of Kidney Necrosis Produced by Chloroform

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Richard V. Branchflower	Staff Fellow	LCP	NHLBI
Other:	Lance R. Pohl	Pharmacologist	LCP	NHLBI
	John W. George	Chemist	LCP	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme-Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.65	0.45	0.20

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Chloroform ($CHCl_3$) was significantly more potent in producing kidney necrosis and depleting kidney glutathione (GSH) than was deuterium labeled chloroform ($CDCl_3$). $CHCl_3$ also produced a greater decrease in the level of kidney GSH in DBA mice, which are more sensitive to the kidney toxic effect of $CHCl_3$ than it did in the insensitive C57 mice. These results indicate one of the rate determining steps in the kidney necrosis produced by $CHCl_3$ is the metabolism of its C-H bond. This finding is consistent with $CHCl_3$ being biotransformed into the toxic metabolite phosgene ($COCl_2$) in the kidney. This metabolite can possibly react with vital tissue components to produce necrosis or be detoxified by reacting with GSH to yield diglutathionyl-dithiocarbonate (GSCOSG).

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PHS-6040
(Rev. 2-81)

Project Description:

Objective: To determine the mechanism of chloroform induced necrosis of the cortical proximal tubules.

Methods Employed: Chloroform ($CHCl_3$) and deuterium labeled chloroform ($CDCl_3$) were administered intraperitoneally at various doses to DBA and C57 male mice. After various times, kidney glutathione levels were measured with Ellman's reagent and kidney necrosis was evaluated by light microscopy examination of kidney sections and by determining the level of blood urea nitrogen.

Major Findings: $CHCl_3$ was significantly more potent in producing kidney necrosis and depleting kidney GSH than was $CDCl_3$. $CHCl_3$ produced a greater decrease in the level of kidney GSH in DBA mice than it did in C57 mice.

Significance to Biomedical Research and Program of the Institute: Although it has been known for over a century that $CHCl_3$ causes kidney necrosis in man and animals, the molecular basis for this toxicity has been elusive. It is known, however, that genetics are important in this toxicity since some strains are particularly sensitive such as DBA males, whereas C57 males are more resistant. Moreover female mice appear to be relatively resistant to the kidney toxicity of $CHCl_3$.

The results of the present study represent the strongest evidence that a metabolite of $CHCl_3$ produces the kidney damage. This follows from the observation that $CDCl_3$ was less nephrotoxic than $CHCl_3$, which indicates that the metabolic cleavage of the C-H bond of $CHCl_3$ is one of the rate determining steps in the toxicity process.

Previous studies with rat liver have shown that the major if not only metabolic transformation of $CHCl_3$ was its oxidative dechlorination to the hepatotoxin, phosgene, $COCl_2$; the breakage of the C-H of $CHCl_3$ was one of the rate determining steps in this process. Therefore if $COCl_2$ was also produced from $CHCl_3$ in mouse kidneys, its rate determining formation could explain why $CHCl_3$ is more nephrotoxic than $CDCl_3$. The finding that $CDCl_3$ depleted less kidney GSH than $CHCl_3$ supports this idea, since $COCl_2$ has previously been shown to react with GSH to produce diglutathionyl-dithiocarbonate (GSCOSG) and less $COCl_2$ should be produced from $CDCl_3$ than $CHCl_3$. This mechanism might also explain why $CHCl_3$ depleted more GSH in the more sensitive DBA mice than in C57 mice. Thus it appears that the metabolism of $CHCl_3$ to $COCl_2$ by kidney cells may be the basis of $CHCl_3$ induced kidney toxicity.

Proposed Course of Project: We intend to 1) determine whether mice kidneys can metabolize $CHCl_3$ to $COCl_2$, 2) characterize the enzymes responsible for this metabolic transformation, 3) determine whether differences in the metabolism of $CHCl_3$ to $COCl_2$ are responsible for the genetic and sex differences in the susceptibility to kidney toxicity produced by $CHCl_3$ and 4) elucidate how metabolite(s) of $CHCl_3$ cause kidney necrosis.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00954-01 LCP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Studies on the Formation and Metabolism of Bromobenzene-Glutathione Conjugates.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Terrence J. Monks	Vist. Associate	LCP	NHLBI
Other:	James R. Gillette	Chief	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme-Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.25	0.25	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have previously isolated and identified two glutathione conjugates of bromobenzene as trans-3-bromo-6(glutathion-S-yl)-cyclohexa-2, 4-dien-1-ol (BB-GSHA) and trans-4-bromo-6(glutathion-S-yl)-cyclohexa-2, 4-dien-1-ol (BB-GSHB). The two conjugates are formed in unequal amounts in a ratio of BB-GSHA: BB-GSHB of 1.6:1. We have therefore purified the various forms of glutathione-S-transferase enzymes from rat liver and examined their ability to catalyze the reaction of bromobenzene-3, 4-oxide with glutathione. All forms of the enzyme isolated exhibit catalytic activity towards the epoxide. However, the ratio of the two conjugates formed with the purified enzymes varies from the ratio obtained both in vivo and with rat liver microsomes and 100,000 x g supernatant. The combined conjugates are metabolized by γ-glutamyl transpeptidase at apparently identical rates, and give rise to five products. One of the products is less water soluble than the starting substrates and might be the dehydrated, aromatized cysteinyl glycine conjugate.

PHS-6040
(Rev. 2-81)

Project Description:

Objective: The two bromobenzene-glutathione conjugates previously described by this laboratory are formed by rat liver microsomes and 10,000 x g supernatant, and excreted into bile in vivo, in unequal amounts. The present study was designed to investigate the biochemical mechanism responsible for the apparent stereospecifity in conjugate formation and/or excretion.

Methods Employed: The formation of the bromobenzene-glutathione conjugates has been shown to be dependent upon the presence of the glutathione transferase enzymes, present in the 100,000 x g rat liver supernatant. Thus the different forms of glutathione transferases were purified by published procedures to ascertain possible differences in their ability to catalyze the conjugation of glutathione with bromobenzene-3, 4-oxide. Such differences might explain the preferential addition of glutathione to the 4-position rather than the 3-position of the epoxide. Another possible reason for the difference in the amounts of the two conjugates might be in their susceptibility to further metabolism. Therefore the two conjugates were incubated with γ-glutamyl transpeptidase to investigate the above possibility.

Major Findings: Glutathione transferases A,B,C, and D + E, all catalyze the formation of bromobenzene-glutathione conjugates. However, transferases A, B, and C all produce more of BB-GSH B than BB-GSH A, in contrast to results obtained in vivo and in vitro with 100,000 x g supernatant. However, glutathione transferases D + E combined produce the conjugates in the same ratio as in observed in vivo and in vitro with 100,000 x g supernatant.

Incubation of the combined bromobenzene-glutathione conjugates with γ-glutamyl transpeptidase yields at least five products. The products have not been characterized. However, one of the products has a long retention time on HPLC and may be the dehydrated, aromatized cysteinyl-glycine conjugates. Moreover, the rate of metabolism of the two glutathione conjugates by γ-glutamyl transpeptidase was identical. The covalent binding of the 3,4-epoxide to the glutathione transferases does not affect their catalytic activity.

Significance to Biomedical Research and Program of the Institute: The differences in the rate of formation and/or excretion of the two bromobenzene-glutathione conjugates derived from bromobenzene-3, 4-oxide may have important implications. The rate of detoxification of chemically reactive metabolites is important in limiting their potential as toxic agents. Therefore elucidating and understanding those factors which influence the detoxification of reactive metabolites is of immediate importance.

Proposed Course of Study: We intend to characterize the products derived from the two bromobenzene-glutathione conjugates when incubated with γ-glutamyl transpeptidase. Moreover we intend to investigate further the discrepancy between the rates of formation of the two glutathione conjugates in vivo and in microsomes supplemented with 100,000 x g supernatant, and their formation in microsomes supplemented with purified preparations of the glutathione transferase enzymes.

Publications: Monks, T.J., Pohl, L.R., Gillette, J.R., Hong, M., Highet, R.J., Ferretti, J.A., and Hinson, J.A.: Stereoselective formation of bromobenzene glutathione conjugates. Chem. Biol. Int., (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00955-01 LCP

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

The mechanism of formation of the phenolic metabolites of bromobenzene

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Terrence J. Monks	Vist. Assoc.	LCP	NHLBI
OTHERS:	Serrine S. Lau	Vist. Fellow	LCP	NHLBI
	Lance R. Pohl	Pharmacologist	LCP	NHLBI
	James R. Gillette	Chief	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Drug Tissue Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS: 0.5	PROFESSIONAL: 0.5	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It has previously been established by this Laboratory that the formation of p-bromophenol from bromobenzene occurs via bromobenzene 3,4-oxide, but o-bromophenol could arise either via the 2,3-epoxide or the 1,2-epoxide, or by direct insertion of oxygen. Using 2,4,6-trideuterobromobenzene we have shown that o-bromophenol arises predominantly from the 2,3-epoxide intermediate although the addition of oxygen to the 2-position carbons, followed by an NIH shift and rearrangement, cannot be ruled out.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: The reactive metabolite of bromobenzene is believed to be the 3,4-epoxide. We have shown that most of the 3,4-epoxide is converted to two glutathione conjugates by the glutathione transferases in the liver soluble fraction. The major urinary metabolite in rats treated with 3-methylcholanthrene is o-bromophenol and it has been assumed that its postulated precursor, bromobenzene 2,3-oxide, is not toxic.

While the formation of phenols from aromatic hydrocarbons is widely accepted to occur via arene oxide intermediates other mechanisms of substrate hydroxylation, without the involvement of intermediate arene oxides, are possible. In view of the apparent toxicity of the pathway leading to p-bromophenol and the nontoxicity of the pathway leading to o-bromophenol, and the apparent lack of formation of a glutathione conjugate from the postulated 2,3-epoxide, the present study was initiated to elucidate the mechanism of formation of o-bromophenol and to determine its relationship to the apparent nontoxicity of this pathway.

Methods Employed: The in vitro formation of p- and o-bromophenol in rat liver microsomes from H-bromobenzene and D-bromobenzene was determined using an HPLC asay developed in this Laboratory. Deutero-orthobromophenol was isolated for mass spectral analysis and its GCMS compared with that of authentic o-bromophenol.

Major Findings: No significant deuterium isotope effect was observed in the formation of either o- or p-bromophenol. The rate of formation of p-bromophenol from H-bromobenzene and D-bromobenzene was 1.00 $\pm$ 0.08 nmoles/mg/min and 1.17 $\pm$ 0.07 nmoles/mg/min respectively (K_H/K_D = 0.85). The rate of formation of o-bromophenol from H-bromobenzene and D-bromobenzene was 0.73 $\pm$ 0.02 nmoles/mg/min and 0.74 $\pm$ 0.06 nmoles/mg/min, respectively (K_H/K_D = 0.99). The GCMS of the o-bromophenol isolated from an incubation of 2,4,6-trideuterobromobenzene showed that 70% of the product retained all three deuterium atoms. The results are consistent with the view tht o-bromophenol is formed via an epoxide intermediate, and that it is formed predominantly from the 2,3-epoxide rather than the 1,2-epoxide. However, the addition of oxygen to the 2-position carbons, followed by an NIH shift and rearrangement cannot be ruled out.

Significance to Biomedical Research and to the Program of the Institute: The fact that the pathway leading to p-bromophenol is toxic while the pathway leading in o-bromophenol formation is not poses several questions. The intermediates in both pathways have been postulated to be epoxides. The inference that one epoxide is toxic while the other is not, illustrates the differences in reactivity between structurally similar chemically reactive metabolites. Such differences must be taken into account when extrapolating from in vitro mechanistic studies to in vivo toxicity studies.

Proposed Course of Project: We intend to determine the relative contribution of the epoxide mechanism and the addition/rearrangmeent mechanism to the formation of o-bromophenol. Moreover, we intend to investigate which of these two mechanisms are responsible for the covalent binding observed during the formation of o-bromophenol from bromobenzene.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00956-01 LCP

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Pathways of inflammatory response in different experimental models in rat

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Theresa N. Lo	Research Chemist	LCP	NHLBI
OTHERS:	Michael A. Beaven	Deputy Chief	LCP	NHLBI
	Elizabeth Wolde-Mussie	Staff Fellow	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Cellular Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS: 1.0	PROFESSIONAL: 1.0	OTHER: 0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A study of the inflammatory reaction following the injection of dextrans (60 mg) of various molecular weights cpd. 48/80 or antiserum to IgE (anti-IgE) into the rat pleural cavity revealed several distinct responses. The dextrans induced accumulation of fluid with little protein and few neutrophils. The exudate induced by cpd. 48/80 contained fluid and plasma proteins. Both responses were of rapid onset (30 min) and were accompanied by mast cell degranulation. Antiserum to IgE also induced mast cell degranulation and rapid accumulation of fluid and protein, and at a later stages (4h) an infiltration of neurotrophils. All these responses were unaffected by indomethacin. The response to anti-IgE was partially blocked by treatment with either metiamide or mepyramine (a H_2 and H_1 receptor antagonist respectively) and completely blocked when both drugs were given simultaneously. Fluid volume and protein content were reduced to a simiextent (99% and 91%, respectively), whereas leukocyte infiltration was reduced by only 46%. These and our previous studies suggest that inflammatory infiltrates may be formed through two distinct mechanisms: one associated with transudation of protein-free fluids, the other to exudation of plasma proteins.

380

Project Description

Objectives: Our previous studies indicated that dextran and carrageenan evoke quite different inflammatory responses. Injection of dextran into the pleural cavity of rat resulted in inflammation of rapid onset to produce within 30 min an accumulation of fluid which contained few neutrophils and little protein. Degranulation of mast cells and histamine release accompanied this response. The response to carrageenan was characterized by a slow but progressive accumulation of fluid, protein and neutrophils. Mast cells remained intact and no histamine release could be detected. The response to carrageenan but not dextran was blocked by indomethacin and other nonsteroidal drugs in a dose-dependent fashion. The amount of fluid, protein and number of white cells were highly correlated and all three parameters were reduced to an equal extent by indomethacin. As described in another report, the protein exudate which was of plasma origin and contained a neutrophil chemotactic factor which was thought to be derived from complement components.

Two (or more) types of mechanism might thus exist for formation of inflammatory exudates, i.e. transudation with the formation of protein free ultrafiltrates and exudation with leakage of plasma proteins. It is also likely that these processes involve different mediators: histamine or mast cells derived products in the case of transudation and cyclooxygenase derived products in the case of exudation. To explore these possibilities further we have compared the inflammatory responses to several mast cell degranulating agents namely, compound 48/80, dextran and antiserum to IgE (anti-IgE). The latter compound has not been studied previously in vivo. The effects of antihistamine drugs and indomethacin were also studied. As in previous studies histamine release, fluid formation, protein and white cell infiltration were monitored. Studies with a nondegranulating agent, carrageenan, are described elsewhere.

Methods Employed: Preparation of materials for injection and processing of pleural exudates. The dextrans (6% to 30% w/v) and compound 48/80 (0.5% w/v were dissolved in normal saline. The dextrans tested included T10,T40, T70,T150,T500, and T2000 (Pharmacia). Lyophilized antiserum to IgE (Miles Laboratory) was reconstited in water and serial dilutions were made in saline. Indomethacin (5 and 10 mg/kg) was injected i.v. Mepyramine (5 mg/kg) and Metiamide (100 mg/kg) were injected subcutaneously 30 min before the injection of the inflammatory agents. Rats were lightly anesthetized with ether, and the inflammatory agents injected into the pleural cavity at thirty min or 4 hr later, rats were killed and pleural exudates were collected and processed as described in Project Z01 HL 00617-06 LCP except that the cavity was washed with 5 mM EDTA-Dulbecco's phosphate buffered saline without Ca++ and Mg++ to minimize clotting of exudates. Formation of fibrin-like clots were observed with in most exudates.

Analysis of histamine and protein. Histamine was assayed by the enzymatic isotopic assay. Samples were diluted in 0.05 M sodium phosphate buffer, pH 7.9, to give an approximate histamine concentration of 10^{-6}M. Cell suspensions were disrupted by sonification (Kontes ultrasonicator at maximum setting for 15 sec.).

Protein was assayed in the supernatant (cell-free) fractions by the method of Lowry et al. The various classes of proteins were identified in the cell-free extract by the double immunodiffusion (Ouchterlony) technique.

Major Findings: Exudate composition 30 min after the injection of inflammatory agents. The dextrans tested include T10,T140,T120,T150,T500,T2000 (Pharmacia) and T250 (Sigma Chemical Co.). The last was found to be contaminated with trichloroacetic acid-soluble peptide material. Accumulation of fluid (0.5-1.0 ml) was evident 30 min after the injection of all agents. With the dextrans, the number of white cells (4-8 x 10^6 cells) and protein (2-8 mg) recovered from the pleural cavity was similar to that obtained by washing the pleural cavity of untreated rats. After treatment with compound 48/80 and anti-IgE, protein increased to 23 mg and 40 mg, respectively, though cell counts remained low.

Although the histamine content of the cavity fluid and washings was high (between 9 and 19 µg), less than 1% of this histamine was recovered in the extracellular fluid of rats treated with saline. Extracellular levels were increased significantly (0.04 to 0.26 µg/ml after the injection of the agents, and cellular levels declined by 3.5% after dextran, 68%, after anti-IgE and over 90% after compound 48/80. This decrease suggested that histamine release was more extensive with compound 48/80. This was confirmed by morphological examination of the exudates. All mast cells remained intact in rats treated with either NaCl or carrageenan. After T10, 35% of the mast cells showed signs of partial degranulation and, after T2000, 58% of the mast cells were degranulated to varying extents. Similar correlation between degree of mast cell degranulation and extent of histamine release was observed in samples collected from anti-IgE treated rats. Few intact mast cells (< 2%) were observed in exudates of 48/80-treated rats.

Dose response curves were determined for all agents. Unlike the response to carrageenan protein concentrations and volume showed no correlation.

Exudate compositions 4 hr after the injection of inflammatory agents. Four hours after the injection of dextans, the exudates still contained little protein (<9 mg) and few cells (<12 x 10^{-6}) with the exception of the highest molecular weight of dextran tested (T2000, Pharmacia) in which small increases in cells (19 x 10^{-6}) and protein (19 mg) were observed. Additional increases in fluid volume (0.7 ml) and protein content (30 mg) were evident 4 hr after the injection of compound 48/80 though cell counts remained low. In anti-IgE treated rats, fluid and protein accumulation appeared to have reached maximum by 30 min as exudate volume (0.8 $\pm$ 0.2 ml) and protein (44 $\pm$ 4 mg) at 4 hr remained unchanged. Significant infiltration of neutrophils (34 $\pm$ 4 million) was, however, evident by 4 hr. A decrease in mast cells (-75%) and no change in numbers of eosinophils and monocytes were also noted.

Study of dose-response relationships again revealed that although protein and white cell counts increased with increasing doses of anti-IgE, they did not do so in a parallel fashion. Furthermore, irrespective of dose or time, protein was observed in all compound 48/80-induced exudates but significant neutrophil infiltration was not been observed.

Effect of indomethacin. The response (30 min and 4 hr) to dextran (4 experiments), compound 48/80 (2 experiments) and anti-IgE (3 experiments, n of 5-10 for each experiment) was unaffected by indomethacin given in doses of 5 and 10 mg/kg i.v. Typically, for the anti-IgE experiment, the exudates contained 0.66 ± 0.09 ml fluid, 34 ± 4 million cells and 41 ± 4 mg protein (n = 18) and, in indomethacin (10 mg/kg) treated rats, 0.66 ± 0.09 ml, 43 ± 2 million cells, 42 ± 4 mg protein. This is in contrast to the consistent inhibition of the response to carrageenan where all exudate constituents are reduced by approximately 30, 55 and 75% with 2.5, 5.0 and 10 mg indomethacin/kg (total of 9 experiments).

Effect of metiamide and mepyramine on the inflammmatory response to anti-IgE. Metiamide (100 mg/kg sc) or mepyamine (5 mg/kg sc) administered 30 min before the intrapleural injection of anti-IgE partially suppessed (40-50%) and completely suppressed accumulation of fluid and protein (94% and 91% respectively) when given in combination. However, the infiltration of neutrophils was decreased by only 20-40%. Inhibition was evident both 30 min and 4 hr after injection of anti-IgE.

Inflammatory response to histamine agonists. In view of the dramatic affect of the antihistamine drugs, the inflammatory response to histamine, the histamine H_1 receptor agonist; pyridylethylamine, and H_2 receptor agonist, impromidine were tested alone and in combination. Even in high doses, none of these produced an intense response (maximum increase in fluid volume and protein were 0.13 ml and 4.8 mg). This is consistent with previous reports that histamine has weak inflammatory action in rat.

Identity of soluble components of the inflammatory exudate. The dextran-induced exudates contained little protein (generally similar to that found in saline washes of the cavity), but did contain inorganic anions and cations in concentrations virtually identical to that in plasma. In the compound 48/80-induced exudates, all of the major classes of plasma protein were identified by the double immunodiffusion technique. Anti-IgE-induced exudates have not been tested.

Significance to Biomedical Research and the Program of the Institute: Anti-IgE, dextran, compound 48/80 and carrageenan-induced inflammation appear to be useful models to study the effects of drugs on different types of inflammatory reactions. With these models, we have shown that mast cell degranulation (and early edema formation) and neutrophil accumulation are unrelated phenomena, although both may occur in response to a single agent. The production of an exudate almost devoid of protein, on the one hand, and of exudate with a protein composition almost identical to that of plasma, on the other, suggests that our understanding of the mechanisms responsible for increased vascular permeability is incomplete and cannot be explained by a single process (opening of capillary pores, for example) or single mediator. The data provide an explanation as to why anti-inflammatory drugs are effective in some forms of inflammation and not in others. The dramatic effect of antihistamine drugs on the inflammatory response to anti-IgE and the weak inflammatory response to histamine agonists suggest that histamine, may act synergistically with other mediators.

Proposed Course of Project: The contribution of histamine release (and other mast cell constituents) to early edema (fluid) response and activation of complement and arachidonic acid metabolites to neutrophil accumulation will be assessed in studies with H_1 and H_2 receptor antagonists, antibodies to components of the complement system, and inhibitors of the lipoxygenase and cyclooxgenase systems.

Publications:

Bayer, B.M. and Beaven, M.A.: Cytostatic activity of pharmacological concentrations of indomethacin in cell cultures and inactivity of closely related compounds. Biochem. Pharmacol. 30: 807-809, 1981.

Bayer, B.M., Almedia, A.P., and Beaven, M.A.: Inhibition of the expression of the "A" system of amino acid transport by anti-inflammatory drugs during cell culture growth and mitogenic stimulation of thymus lymphocytes. J. Pharmacol. Exp. Ther. 219: 752-759, 1981.

ANNUAL REPORT OF THE
LABORATORY OF CHEMISTRY
SECTIONS ON CHEMICAL STRUCTURE AND STRUCTURAL
NUCLEAR MAGNETIC RESONANCE
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

October 1, 1981, through September 30, 1982

The Laboratory, as usual, consists of two quite differently organized groups. One, the Section on Physiological Chemistry under Dr. John Pisano with 8 full and part-time professionals is concerned with the isolation, identification and function of physiologically important peptides; the report of his Section is appended. The second, consisting of the Sections on Chemical Structure and Nuclear Magnetic Resonance under myself and Dr. Robert Highet is concerned with isolation, elucidating the structures, and studying the properties of biologically important compounds.

As in past years, our focus is on mass spectrometry, usually the first tool used in identification of an unknown, nuclear magnetic resonance which defines not only structure but solution dynamics (conformation, interaction with surfaces, etc.), and finally, X-ray crystallography which is used when a sufficiently purified sample is available and fine details of structure are required. This year a new and hopefully increasing dimension, that of organic synthesis, has been added in the form of Dr. Norman Schmuff. This activity replaces our interest in the computer organization of scientific data, an activity under Dr. G. W. A. Milne that although still critical to the overall scientific community, requires resources beyond those available to us and is more properly supported by the extramural program and NSF.

In mass spectrometry, the ^{252}Cf plasma desorption spectrometer built for us by Texas A&M has finally arrived but a few problems exist in interfacing with our newly acquired P.E. 3220 computer. These are being addressed by Dr. Ramon Tate (DCRT) and the system is expected to be operational in 1-2 months. The system has been used in Texas to confirm the structures of several tryptophan containing tripeptides, a mixture of digitoninethylene oxide and propylene oxide adducts, encephalin-related peptides, etc. A fast atom bombardment system built for the MS-9 by Dr. L. Kelner (BEIB) was not immediately successful due to arcing, but newer developments prove that ions can replace atoms in this application, so a simplified cesium ion gun will be built and installed shortly. This system should be an ideal complement to our Cf system for high molecular weight, polar and sensitive compounds. Computerization of the MS-9 is now proceeding on schedule due to the interest of one of our summer students, Randy Hill.

In nmr spectrometry, the Varian HA-100 has finally been surplussed (to NRL where the magnet will find another use), because its electronics and data system could not economically be repaired or serviced. The Nicolet high field supercon is increasingly in demand by biochemists, but it has had a disappointly high downtime. It is apparent that the Nicolet Company does not have suitable maintenance personnel, and we will have to be increasingly self-reliant in this respect. Fortunately, Dr. Jim Ferretti is now formally attached to our laboratory and in addition to another new dimension he brings to the laboratory (biophysics),

his assistance in supervising this instrument will be important. It is becoming clear, however, that because these biologically-oriented experiments are so popular and take so much instrument time, the development of new nmr techniques, such as that developed by Dr. R. J. Highet in characterizing trisubstituted double bonds, the analysis of routine lab samples, and other experiments more typical of our laboratory's interest, is impeded. A more routine, somewhat less sophisticated spectrometer may become necessary in the future but the ideal instrument is not apparent now.

In X-ray crystallography, Dr. J. Silverton in ever-increasing activity, has completed twelve structures this year, including such compounds as the important antitumor antibiotic, frederickamycin, viridicatum toxin, and the cardiac glycoside, humistratin. It is clear that he requires more room and he will shortly move into an expanded module. A newly acquired videochart has been applied to the early stages of the crystallographic technique and appears to be very useful.

Alone or in collaboration (see individual reports for identification of collaborators), members of the laboratory this year have: 1) separated by HPLC and identified a potent molluscicide (6-pentadec-8,11,14-trienyl) salicylic acid) from Anacardium occidentale, 2) identified a new carcinogenic pyrrolizidine alkaloid from Senecio glabellus, 3) completed GLC-MS studies on the Israel weaver ant (Polyhachis simplex), 4) identified methylheptenones and hydroxymethylpentanone in glandular extracts of Tapinoma simrothi, 4) identified a new dodecenoic acid in Bryomyrmex syriacus; its double bond position is under study, 5) developed an HPLC method for analyzing oligosaccharides derived from glycoproteins; an improved detector (TECATOR) is necessary for sufficient sensitivity and will be acquired, 6) developed a new method for characterizing trisubstituted double bonds by (a) pulsing at appropriate ^{13}C frequencies, (b) developing processions with proton coupling, (c) turning off the coupling and (d) collecting the free induction decay; the technique has been demonstrated with several molecules including citraconic anhydride and dimethylacrylic acid, 7) using intramolecular Overhauser effects, established the stereochemistry of the sugar moiety of chrysomycin as 6-deoxy-7-methylgulose, 8) located the double bond in the hyena lactone (14-hydroxy-12-tetradecenoic acid), 9) studied restricted rotation of the formamide moiety in a series of bromoformylretuline used in a new synthesis of morphine, 10) solved the crystal structures of t-BOC-$(Gly)_3$-OBenzyl, 11) solved the structures of a sinomenine dimer, N-formyl norreticuline (showing only one conformer in the solid state, two in solution), 12) elucidated the nature of hydrogen bonding in the frog poison histrionicotoxin, 13) solved the structure of desoxadiol to study its interaction with DNA, 14) elucidated the structure of a novel antitumor antibiotic frederickamycin of nearly 1000 Daltons in the assymetric unit, 15) solved the structure of a multi-ring (3,5,5,6,7) chemotherapy drug, 16) determined the absolute configuration of viridicatum toxin, 17) determined the double bond location in humistratin, 18) discovered that polyphosphates are partially sequestered in inclusions in Corynebacterium during some phases of cell growth, accounting for their failure to provide NMR signals; calcium and magnesium ions play a role in their hindered mobility, 19) observed fluorine nmr signals from 6-fluorodopamine in nerve microsacs from guinea pig brain and identified signals from two of its metabolites, 6-fluorodihydroxyphenylacetic acid and 6-fluorodihydroxyphenylethanol, 20) discovered two new compounds 3-heptene-2,5-dione and 3-nonene-2,5-dione in Trigona, the killer bee killer (these are new natural

products), 21) identified and synthesized four new metabolites of the anti-molluscicide, amoscin, 22) completed a comparative study of the attack (sting) pheromones of four Apis species, including the killer bee, 23) identified the reaction product of digitonin and ethylene oxide (and propylene oxide) as a series of compounds with up to 6 oxide units (one for each primary hydroxyl) per molecule of digitonin, 24) completed a study of fifteen encepholine-related peptides by ^{252}Cf plasma desorption mass spectrometry, 25) elucided the structures of a series of reaction products of oligosaccharides (from glyco-proteins) with anisole as 1,1 dianisylidensaccharides, 26) the Spanish toxic oil syndrome has been investigated; samples of the oil were found to contain fatty acid anilides presumably from added aniline--these compounds were not the source of the toxicity, however, 27) Mesophonera species were found to contain N-amyl-N-nonylamine and its N-formyl derivative; 28) several new analogues of nifedipine have been synthesized for affinity chromatography, 29) the structure of a xanthenoxanthein derivative related to the mold metabolite elsinochrome, has been elucidated, 30) a series of synthetic products related to hypusine have been studied, 31) an extract of Scylla lillio-hyacynthus L., reported to be hypotensive, has been purified and studied; only hypertensive activity was found, 32) a new stereocontrolled synthesis of Δ^3-unsaturated esters has been developed, 33) a new stereospecific synthesis of allylsilanes has been developed.

ANNUAL REPORT OF THE
LABORATORY OF CHEMISTRY
SECTION ON PHYSIOLOGICAL CHEMISTRY
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1981, to September 30, 1982

The Section on Physiological Chemistry is concerned with biologically active polypeptides including their discovery, biosynthesis, and mode of action. Two projects continue to be of major interest: the kallikrein-kinin system, and the discovery and characterization of new biologically active peptides.

Kallikrein-Kinin System

Bradykinin is one of the most potent vasodepressor and pain-producing substances known. Its role in the regulation of blood pressure in normal man is unclear but in certain pathologic states it can have a profound effect. In blood, bradykinin is formed from the protein kininogen by the enzyme kallikrein. Kallikrein also occurs in many exocrine glands including the kidney. Glandular kallikrein has different physico-chemical properties from plasma kallikrein. The kallikrein-kinin system is believed to play an important role in the regulation of renal tubular water and electrolyte transport and this year's efforts have centered on the kidney.

Distribution of Immunoreactive Kallikrein Along the Rat Nephron: To obtain more convincing evidence that kinins have a role in renal transport, we have studied the distribution of cellular kallikrein along the nephron. Using a direct radioimmunoassay, we have localized kallikrein along the cortico-medullary axis of the rat kidney and in microdissected rat cortical nephron segments. Kallikrein content was highest in the outer cortex and decreased progressively toward the papillary tip. The kallikrein content in the cortex was significantly increased following dietary sodium restriction. In microdissected cortical nephron segments, the highest kallikrein content was found in the connecting tubule. A significant amount of kallikrein was found in distal convoluted tubules, initial collecting tubules, and cortical collecting ducts. Little or no kallikrein was found in glomeruli, proximal convoluted tubules, proximal straight tubules, and cortical thick ascending limbs. These results are compatible with the site of action of the kallikrein-kinin system in the cortical segments of the rat distal nephron.

The Kallikrein-Kinin System in Rat Renal Lymph: We have extended our previous studies (Z01 HL 01016-11C) on renal lymph to include a comparison with thoracic lymph. Renal lymph contains immunoreactive glandular kallikrein and like the immunoreactive glandular kallikrein in plasma, it is biologically inactive. While the kallikrein level in renal lymph appears to be similar to that in thoracic lymph, renin levels are 400-fold those of thoracic lymph and 50-fold those of plasma. In contrast, renal lymph contains approximately one-tenth as much angiotensin converting enzyme as either plasma or thoracic lymph and significant levels of AI. Thoracic lymph contains undetectable levels of AI. It appears that while renin is elaborated into the circulation by the kidney, this organ is not a major source of the immunoreactive glandular kallikrein in plasma. Our results so far do not, however, allow us to exclude the possibility

that part of the immunoreactive kallikrein in lymph orginated from the tubular cells and was initially biologically active. Renal lymph also contains kinin and kininogen. The latter was determined by incubation of lymph with kallikrein.

Kinin Receptors in the Kidney: To gain a better understanding of the role of the kallikrein-kinin system in the kidney, it is necessary to know which components (kallikrein, kininogen, kinins, kininases) occur in this organ, their distribution, their site of interaction and the localization of kinin receptors. Initial studies on kinin receptors have shown that freezing of membrane fractions does not significantly alter specific binding of [^{3}H]-bradykinin. This information indicated the feasibility of extending the study to segments of microdissected nephrons where three days are required to obtain enough material for study. Daily collections were stored at -80° until tested (usually on day eight). Specific binding of [^{3}H]-bradykinin to membrane preparations from homogenized medulla was 8-fold higher than cortex. Microdissected nephron segments examined were proximal convoluted tubule and the length from the thick ascending limb of Henle's loop to the collecting tubule. No specific binding was detected in the proximal convoluted tubule but binding was found in the length from the thick ascending limb to the collecting tubule.

The Kallikrein-Kinin System in the Isolated Perfused Rat Kidney: The isolated perfused kidney is an useful model to study the role of the kallikrein-kinin system in this organ. It was previously known that this preparation elaborates kallikrein into the perfusate and into the "urine". We have extended these studies to an analysis of the "urine" for kinin and kininogen to determine if all the necessary components are present and active. The preparation clears inulin normally and does not show any evidence of tubular damage during the first hour. Significant levels of free kinin are produced and additional kinin is released after incubation with trypsin. This additional kinin evidently comes from kininogen. Only one third as much kallikrein is produced if the kidney is pretreated with puromycin; kinin and kininogen values do not change. This suggests that kallikrein turnover is very rapid in the rat kidney.

Prokallikrein

Urinary kallikrein largely arises from the kidney. About half the kallikrein in urine is in the form of the proenzyme. This indicates the occurrence of an activating enzyme which could be a major determinant of active kallikrein levels. In addition to our interest in the putative activating enzyme, we also wish to know the molecular events which accompany this activation of prokallikrein. These studies require an adequate supply of prokallikrein which must be isolated from hundreds of liters of human urine. A major obstacle has been the first step, concentration of kallikrein. We have developed a batch-adsorption step which is much faster, convenient and yields enzyme with higher specific activity than the previously used hollow fiber ultrafiltration.

Peptide Biochemistry

Convinced that numerous peptides with profound biological activities await discovery in man, we have embarked on a program to isolate and characterize peptides from naturally-occurring rich sources, such as insect and animal venom and amphibian skin. The rich sources are logical choices because it is highly

probable that the structure of the new peptides will be closely related if not identical with peptides normally present in minute quantities in human tissues. The rationale includes: (1) development of suitable microprocedures for the isolation and characterization of peptides, (2) development of biological screening tests (a critical step for the discovery of new peptides), (3) production of antibodies to the new peptides, and (4) use of the antibodies for the detection of cross-reacting peptides in man.

Since simple screening tests are vital for the discovery of new peptides, we have compared 41 polypeptides for their ability to lyse liposomes and release histamine from rat peritoneal mast cells. There was a high correlation between the tests but there are notable exceptions. Most active in both tests were bee venom, mastoparan, polistes mastoparan, melittin and gramicidin S. Intermediate activity was observed in the liposome tests with apamin, compound 48/80 eledoisin-related peptide, granuliberin R, polymyxin B, somatostatin, and substance P, but with mast cells, this group was as active as the first group. Interestingly, polistes kinin and calcium ionophore were inactive on liposomes but were about as active as the above peptides in causing histamine release from mast cells.

The liposome lysis test was used to assay chromatographic fractions of yellow jacket (Vespula maculifrons) venom extracts. Two active fractions have been isolated and the amino acid composition of one has been determined. In addition to the second peak in Vespula maculifrons, another peak of activity has been observed in hornet (Dolicovespula maculata) venom.

Procedures have been set up in the laboratory to do amino acid and end-group analyses. The latter are performed primarily by the dansyl procedure using micro-TLC and HPLC for the identification of the dansyl amino acids. Carboxypeptidases A, B, and Y also have been successfully employed. We have found that Hageman factor inhibitor that we isolated from pumpkin seeds (Z01 HL 01018-23CH)) contains N-terminal arginine and C-terminal glycine.

More Gila monster peptide has been isolated and the amino acid composition determined. The N-terminus is histidine and the C-terminus, serine.

The titer of antibody to ranatensin (and the antibody to bombesin) has been increased. This antibody which recognizes neither bombesin nor GRP has been used in the discovery of immunoreactive ranatensin in rat brain. The peptide elutes identically to ranatensin on Sepadex G-25 columns but is resolved from ranatensin into three immunoreactive peaks by HPLC. Interestingly, one peak, but not the other two is recognized by an antibody specific for bombesin. This peak, therefore, has both ranatensin and bombesin antigenic determinants but is identical to neither.

Antibody has been used to visualize ranatensin in neuronal processes by immunocyctochemistry in certain regions throughout the brain and the distribution determined by RIA. The distribution is different from that described for bombesin, a related peptide.

In previous experiments we found that met-enkephalin shifts the dose-response curve of ranatensin making it less potent on the guinea pig ileum. We have now found the converse in rat brain. When morphine or met-enkephalin are injected intraventricularly, strong catatonia appears in 2-3 min. However, when ranatensin was simultaneously injected, catatonia was delayed and did not appear until 10-12 min. The closely related bombesin does not share this morphine-antogonistic property of ranatensin.

Mastoparan is a tetradecapeptide (isolated from wasp venom) noted for its ability to degranulate rat peritoneal mast cells. We have undertaken a study to determine if it, or a related peptide, occurs in higher animals. Antibodies have been prepared against mastoparan and a radioimmunoassay developed using [^{125}I]Tyr-mastoparan. Immunoreactive mastoparan has been detected in rat brain and human urine. Two peaks of urinary immunoreactivity are seen on Sephadex G-25. The first peak elutes with proteins and does not give a line parallel to the standard. The second peak elutes in the peptide fraction and gives a parallel line. Less is known about the brain immunoreactivity.

[^{125}I]-Tyr-mastoparan also has been used to test for binding protein(s) or receptors in rat brain membranes. The labeled material was purified by HPLC and the diiodinated peptide used. Several methodological problems relating to the highly adsorptive nature of the peptide had to be overcome. Binding to rat brain membranes is linear, specific, and saturable. Of numerous peptides tested, only structurally related peptides, melittin and apamin (polypeptides from bee venom) displaced [^{125}I]-Tyr-mastoparan. Binding was also inhibited by mono and divalent cations, the latter being more potent. Bound peptide was largely recoverd unaltered from the membrane fraction. Somewhat unexpectedly, [^{125}I]-Tyr-mastoparan binding was similar with membrane fractions from various brain regions and membrane fractions from various organs. Thus, it appears that binding is not to a specific receptor but to some structure common to all membranes. Since melittin, a well-known membrane-active polypeptide, displaces mastoparan and is known to stimulate phospholipase A_2, a constituent of membranes, the possibility that mastoparan interacts with phospholipase should be considered.

Neuroblastoma-glioma hybrid cell membranes contain a well-characterized receptor for different neuromodulators including met-enkephalin, endorphin, acetylcholine, noradrenaline, adenosine and prostaglandins. Stimulation of these receptors causes an inhibition or stimulation of adenylate cyclase with parallel changes in the production of cAMP. Extracts of 1) Vespula pennsylvanica, 2) Vespula germanica, 3) Vespula maculifrons, 4) Polistes annularis, 5) Dolichovespula maculata 6) Vespa crabro and 7) Gila monster venom were tested. Venoms 1-4 stimulated the cyclase whereas 5,6 and Gila monster were inactive. Venom 4 had the strongest excitatory activity. The active material was isolated and identified as adenoside, a well-known stimulant.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01002-09 CH

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Nuclear Magnetic Resonance of Natural Products

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: E. A. Sokoloski Chemist CH NHLBI

COOPERATING UNITS (if any)

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LAB/BRANCH
Laboratory of Chemistry

SECTION
Structural Nuclear Magnetic Resonance Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

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1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Nuclear magnetic resonance spectroscopy has been applied to intact cells. Polyphosphate inclusions in corynebacterium are examined by phosphorus-31 NMR. Time of growth and cation concentration are found to be important for the resonance to be observable. Nerve microsacs incubated with 6 F-dopamine allow observation of an fluorine resonance from amine and metabolites. C-13 Glucose metabolism study in progress on Giardia cells.

PHS-6040
(Rev. 2-81)

Goal: To explore areas of investigation where application of nuclear magnetic resonance can add vital or previously unavailable information in biochemical research of natural systems.

Introduction: The field of nuclear magnetic resonance spectroscopy, because of the newer more sophisticated instrumentation, provides new problems and also new opportunities to explore biological systems. Intact cells are particularly interesting subjects for NMR investigation. The non-invasive nature of this form of spectroscopy makes it ideally suited for intact cell research. The nuclear probes that can be used are varied, and we have applied mainly fluorine-19, phosphorus-31 and carbon-13 in our current work.

Organic phosphates have been extensively studied by phosphorus NMR; phospholipids and nucleotides, like ATP, are extremely important compounds in life processes. The NMR research to date has shed much light into the form and function of these materials. A little less glamorous but still important group of phosphorus compounds are the inorganic phosphates. Polyphosphates are large, sometimes highly branched, phosphate polymers which have been observed with some difficulty by NMR in living cells. The problem has been that sometimes they are visible by nmr and sometimes they are not,even though other techniques would indicate that substantial amounts were present. In a collaborative effort with Drs. J. Webster and J. Costa (NIMH), we undertook to examine and explain this anomaly. Corynebacterium was used as a model system for our work. The phosphorus NMR signals were found to be present during some phases of the cell growth and not during others. Following the polyphosphate resonance when cells were incubated for various times gave a comprehensive picture of the stages of polyphosphates during cell growth. Observations of cells grown with various concentrations and ratios of calcium and magnesium cations present, or removed during later stages of cell growth with chelex as part of the growth agar, were also very enlightening. Correlation of the NMR data with electron microscopy convinced us that the polyphosphates are sequestered in small inclusions and that the mobility of the polyphosphates is hindered during certain stages of the cell growth. The calcium and magnesium concentrations were also found to play a role in the hindered mobility, while diamines present during cell growth reversed the hindered mobility to a degree. Our finding should help explain the discrepancies found when examining polyphosphates in living cells. Additional work is needed to explain the role of the diamines in increasing the mobility of these polyphosphate inclusions.

A second series of experiments utilized fluorine-19 nuclear magnetic resonance. The absence of naturally occurring fluorinated compounds in biological systems eliminates the difficulties encountered from background interference. The sensitivity of fluorine NMR, second only to proton, allows observation in a reasonable time period with good signal to noise. Nerve microsacs from the stiatum of guinea pig brain were incubated with synthetic 6-fluorodopamine and observed at 4°C in 12 mm NMR cells. We observed resonancs for the 6-fluorodopamine as well as two additional sharper resonances attributed to metabolites. The metabolites are tenatively identified from their HPLC retention times as 6-fluorodihydroxyphenylacetic acid and 6-fluorodihydroxyphenylethanol. Our experiment demonstrated that problems from heterogenity of intact tissues and nerve endings can be overcome, allowing direct observation of biogenic amines

stored in the central neurons. The mere fact that the observation was possible suggests that these amines have considerable molecular mobility within the microsacs. Elevation of the temperature from 4°C to 10°C caused a sharpening of the 6-fluorodopamine peak and eventual disappearance as the metabolism resulted in a final concentration of less than 5% of the original amount as determined by HPLC. These experiments should open new roads of exploration into amines sequestered in living cells.

Other experiments are in progress, using fluorinated and carbon-13 labeled amines in animal and human blood platelets, as well as C-13 glucose in Giarhdia cells.

Publications: none

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01003-10 CH

PERIOD COVERED

October 1, 1981 - September 30, 1982

TITLE OF PROJECT (80 characters or less)

Structure of Natural Products Using Instrumental Methods

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. M. Fales	Chief, Lab. of Chem.	CH	NHLBI
OTHER:	T. Jaouni	Chemist	CH	NHLBI
	N. R. Schmuff	Staff Fellow	CH	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemistry

SECTION

Chemical Structure Section

INSTITUTE AND LOCATION

NHLBI-NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.6	2.6	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

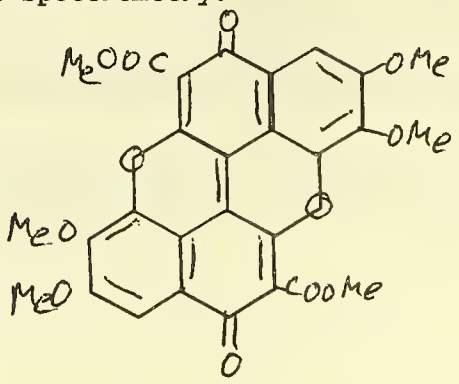
☐ (a1) MINORS ☐ (a2) INTERVIEWS

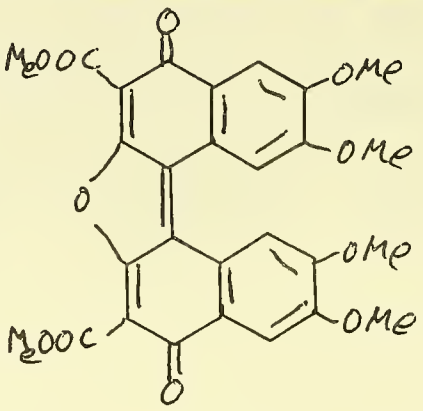
SUMMARY OF WORK (200 words or less - underline keywords)

Two Nitrendipine analogues have been synthesized for affinity chromatography. Samples of oil implicated in the Spanish Toxic Oil Syndrome have been found to contain fatty acid anilides. Anisole-HF reactions with oligosaccharides have been found to to yield 1,1-dianisylidene-mono-saccharides. New stereocontrolled synthesis of Δ^3-unsaturated esters and stereospecific synthesis of allylsilanes have been developed. Intermediate for biosynthetic studies of hypusine have been investigated. Rose furan and perillene have been found in Thripps; N-amyl- and N-formyl nonylamine have been found in Mesopenera species. The structure Xanthenoxanthenequinone related to elsinochrome has been elucidated. 3-Heptene-2,5-dione and 3-nonene-2,5-dione have been found in Trigona.

PHS-6040
(Rev. 2-81)

1) The structure of a red compound (I) obtained from VOF_3 oxidation of 2, related to the elsinochrome mold metabolites, has been deduced from NMR, uv and mass spectrometry.





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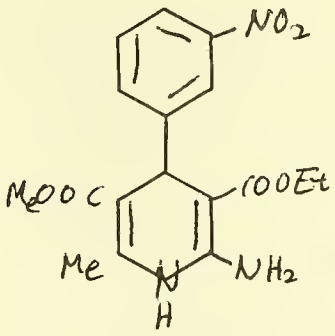
2) Compounds 3 and 4, analogues of nifedipine, have been synthesized for binding to agarose for affinity chromatography of calcium channels (M. Nirenberg, NHLBI).



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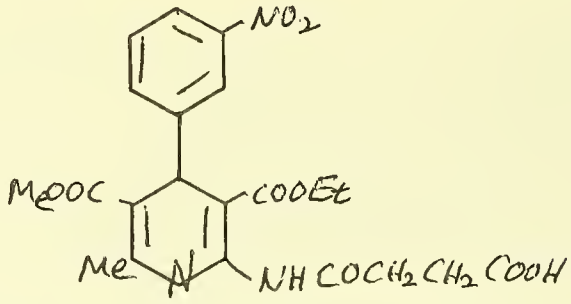
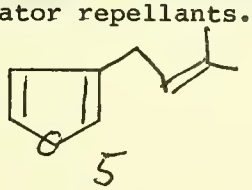


4

3) Samples of salad oil implicated in the Spanish Toxic Oil Syndrome have been investigated by GC-MS and liquid chromatography (H. A. Lloyd, this laboratory). Varying amounts of fatty acid anilides were discovered as contaminents, but these do not seem to be the compounds responsible for the oil's toxicity (R. Kimbrough, CDC, Atlanta). No other toxic principles have been discovered in this or other laboratories. The malady appears to be an immune mediated disease.

4) Larvae of series of Thripps (Teuchothripss, Gynaikothripps and Schedothripps) have been shown to contain perillene (5) and a compound believed to be "rose furan" (6) in addition to hexadecyl acetate. They probably serve as predator repellants.



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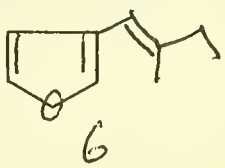
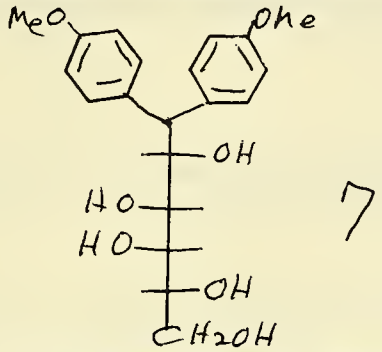


6

396

5) A series of synthetic products involved in the synthesis of ^{15}N labelled spermidine used in studies of the biosynthesis of hypusine (J. Folk, NDI) have been identified using GC-MS and NMR.

6) When anisole and HF are used as solvent and reactant for cleavage of sugars from glycoproteins related to HCG, a series of compounds are formed which are anisole-sugar condensation products (6).

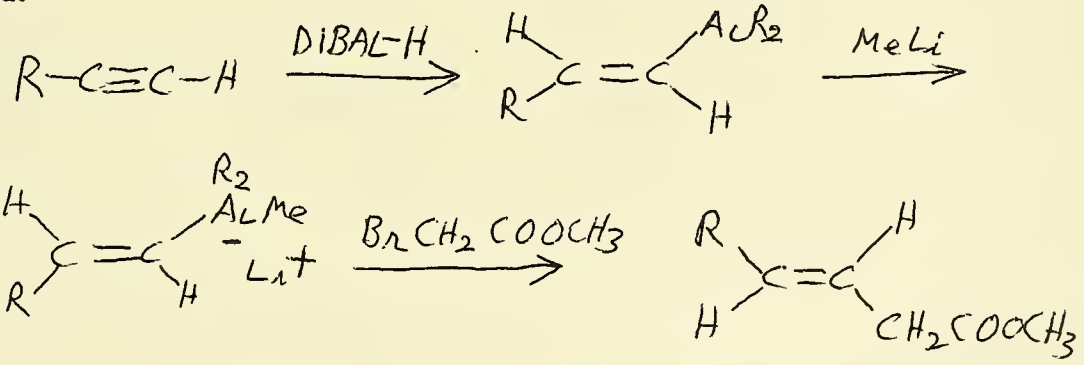


7

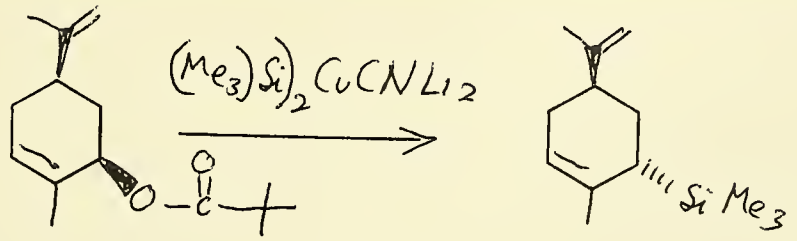
The method seems to have some use in selective cleavage of sugars (Y. Shimohigashi, NICHD).

7) A lilly (Scylla lilio-hyacynthus L) of reputed hypotensive activity (K. Valentin, Israel) has been separated into polar and non-polar fractions by HPLC. Unfortunately, the polar fraction was totally inactive and the nonpolar fraction, hypertensive in SHR rats.

8) A new stereocontrolled synthesis of Δ^3-unsaturated esters has been developed.



9) A stereospecific synthesis of allylsilanes has been developed.



The silanes are useful in reactions with electrophiles such as carbonyl compounds, etc.

10) New Zealand ants (Mesoponera sp.) were found to contain N-amyl- and N-formylnonylamine. These substances are new natural products.

11) Two new unsaturated diketones 3-heptene-2,5-dione and 3-nonene-2,5-dione have been found as part of the pheromone mixture of the killer bee killer, Trigona.

Publications:

1. Kerr, W. E., Blum, M., and Fales, H. M. Communication of food sources between workers of Trigona (Trigona) spinipes. Rev. Basil. Biol. 41: 619-623, 1981.

2. Jones, T. H., Blum, M. S., and Fales, H. M. A facile synthesis of 2-hydroxy-6-methylacetophenone. Synthetic Commun 11: 889-894, 1981.

3. Jones, T. H., Blum, M. S., Howard, R. W., McDaniel, C. A., Fales, H. M., DuBois, M. B., and Torres, J. Venom chemistry of ants in the genus Monomorium. J. of Chem. Ecol. 8: 285-300, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01004-11 CH

PERIOD COVERED
October 1, 1981 - September 30, 1982

TITLE OF PROJECT (80 characters or less)
Characterization of Natural Products

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: H. A. Lloyd Research Chemist CH NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemistry

SECTION
Chemical Structure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Determination of structure of physiologically active compounds from animal or plant tissue by degradation and spectrometric techniques

PHS-6040
(Rev. 2-81)

1. Physiologically active components of Anacardium occidentale (in collaboration with Dr. G. Krishna, CP, NHLBI). Some of the long-chain substituted phenols and salicylic acids isolated from the shells of cashew nuts have interesting properties. The 6-(pentadec-8,11,14-trienyl) substituted salicyclic acid in particular appears to be a potent molluscicide and also acts as a specific inhibitor of prostacyclin receptor in human blood platelets. The feasibility of large-scale separations by high pressure liquid chromatography has been studied to obtain pure samples and study the mechanism of such activity.

2. Plant natural products of pharmacological interest (with Dr. G. Kapadia, Howard University). A number of plant extracts were screened by GC-MS and their constituents identified. New and known pyrrolizidine alkaloids were isolated from Senecio glabellus. Structural determination of these carcinogenic compounds is in progress.

3. Insect pheromones (with Dr. M. Blum, University of Georgia and Dr. A. Hefetz, Tel-Aviv University). Many glandular extracts from ants and bees collected in Israel were screened by GC-MS. Studies on the Israel weaver ant (Polyhachis simplex) were concluded. A comparative study of the secretions of three dolichoderine ants was initiated. The Tapinoma simrothi secretions contained, in addition to 6-methyl-5-hepten-2-one, two less common ketones, 2-methyl-4-heptanone and 4-heptanone, and a product not previously identified in arthropods, 4-hydroxy-4-methyl-2 pentanone.

Another Dolichoderine, Bryomynmex syriacus, does not appear to secrete terpene-derived compounds as most of its glandular secretion consists of a dodecenoic acid. Several microscale reactions did not reveal unequivocally the position of the double bond in this acid. Synthesis of various isomeric dodecenoic acids is being investigated in collaboration with Dr. N. Schmuff of this laboratory. The secretion of a third Dolichoderine ant, Limetopum sp., also appears to contain new or unusual products.

4. Separation of oligosaccharides derived from glycoproteins (in collaboration with Dr. H. Fales of this laboratory and Dr. B. Weintraub, CEB, NIADDK). In view of the ultimate determination of structure of the oligosaccharides by MS, a method for obtaining pure samples, must be devised. High pressure liquid chromatography coupled with a new high sensitivity refractive index detector is being studied. Preliminary results are promising.

5. The Spanish Toxic Oil Syndrome has been investigated and a high pressure liquid chromatographic assay for the contaminating fatty acid analides has been developed.

Publications.

1. Sullivan, J. T., Richards, C. S., Lloyd, H. A., and Krishna, G. Anacardic Acid: Molluscicide in Cashew Nut Shell Liquid. Plant Medica, 44: 175-177 (1982).

2. Hefetz, A., and Lloyd, H. A. Exocrine Glands of Polyrhachis simplex: Chemistry and Function. J. Chem. Ecology, 8: 635-639 (1982).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01005-11 CH

PERIOD COVERED
October 1, 1981 - September 30, 1982

TITLE OF PROJECT (80 characters or less)
X-ray Structural R & D for Physiologically Important Molecules

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. V. Silverton	Research Chemist	CH	NHLBI
OTHERS:	A. Bavoso	Visiting Fellow		NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemistry

SECTION
Chemical Structure Section

INSTITUTE AND LOCATION
NHLBI-NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.3	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Crystallographic studies providing unique or most rapid solutions of structural problems. Development and use of Direct Methods for large molecules.

401

PHS-6040
(Rev. 2-81)

1. Peptides and nucleotides

Considerable work has been carried out in attempts to crystallize linear peptides, particularly those with relation to hypertension. Much work still seems necessary, particularly on purification. It seems unfortunately true that linear peptides, while knowledge of crystal conformation could be valuable, are among the most difficult compounds we have encountered for obtaining crystals. Work will continue since the compounds are not only interesting biochemically, but will offer an opportunity to test some new theoretical and experimental notions of Hauptman for the molecular weight range about 1000 where standard direct methods have difficulties.

In collaboration with Dr Niu (NIADDK), a putative nucleotide-peptide complex was investigated but it quickly became apparent that the crystals were composed only of the peptide: t-Boc-$(Gly)_3$-O-benzyl. Since the crystal structure of the peptide was unknown, it proved worthwhile to do the determination.

2. Analgesics, morphinoids and related drug compounds.

The structure of the dimer of sinomenine is in the final stages of refinement.

The crystal structure of N-formyl-norreticulene has been completed with a view to obtaining information as to its conformation and also to attempt to elucidate the reasons for the known presence of two conformers in solution but only one in the solid state. The reasons are connected with details of crystal packing. A publication is in press.

The nature of the hydrogen bonding and reactivity of histrionicotoxin have been investigated. The structure is in press.

The structure of desoxadiol (with Dr. A. Jacobson, NIADDK) has been completed with a view to investigating its interaction with DNA.

3. Natural and synthetic compounds.

After much work and extended purification, it proved possible to obtain crystals of the antitumor antiobiotic fredericamycin (with Drs. Ramesh Pandey and Renuka Misra, NCI, Frederick). The crystals were not of very high quality and the problem was inherently difficult since the repeating unit is two molecules and represents an asymmetric unit weight of nearly 1000 Daltons. However, the use of direct methods techniques developed previously in this laboratory allowed the answer to be found. The compound proves to have a unique skeleton for an antibiotic. The compound, incidentally, may well have important antitumor activity and is being produced in kilogram quantities for testing. A publication is in press and the structure will also be reported at the 13th International Symposium on the Chemistry of Natural Products, Pretoria, South Africa, August 1982.

A multi-ring compound (3,5,5,6,7) of interest in the synthesis of drugs for chemotherapy (with Dr. Victor Marquez, NCI) has been successfully investigated in order to determine its absolute configuration and to rigorously prove its structure. The crystallographic work is complete.

The absolute configuration of viridicatum toxin has been determined with the help of programs developed here (collaboration with Prof. T. Akiyama, School of Pharmacy, University of Tokyo and Dr. C. Kabuto, Tohoku University). The paper on the structure has been submitted.

The structure of the cardiac glycoside humistratin (with Drs. Nishio and Blum, University of Georgia and Dr. Highet, NHLBI-LC) has been determined to settle the position of the double bond in the steroid. The determination did not seem possible by any other method. The work has been published (see bibliography).

A collaboration with Prof. R. Hill of the University of Georgia has been initiated to investigate some bridged phenanthrenes. The structure of a ketone was determined and work is proceeding on the related hydrocarbon but further investigation will be needed since it appears that the crystals are twinned and currently it is not known if useful information can be obtained about this probably highly strained compound.

4. Antimalarial compounds (collaboration with Drs. D. Klayman and J. Scovill, Walter Reed Institute of Research).

A thiosemicarbazone of potential antimalarial activity has been investigated and the crystallagraphic work is complete. It is interesting that the H atom of the thiosemicarbazone moeity was found in a different place from that expected from the accepted formula.

The Ni complex of the above compound, which is also of interest for antimalarial and perhaps antitumor activity, has also been investigated. The crystal structure is complete and interpretation of the results is in progress. Some preliminary work on a seleno analog was also carried out to ascertain whether isomorphism existed since the differences from the thio compound might be of importance in understanding its biological activity. However, the compound did not prove to be isomorphous and some careful evaluation of the importance of the project will be done before deciding to determine the structure.

5. New equipment.

A video-chart (solid state analog of a chart recorder) was acquired this year and has proven of great value in considerably accelerating preliminary work on crystals. The apparatus has many other features which we hope to fully utilize when the X-ray apparatus is moved to its new location. It is difficult to make definitive plans for new experimental set-ups when one does not know the time at which the move will take place. The move has been "imminent" for one year and is greatly desired since it will provide a little much-needed space for the X-ray activities and also for the computer activities in the laboratory. Low temperature equipment of a new type is being purchased and will be installed after the move.

Publications.

1. Rosner M., Brossi, A. and Silverton, J. V. Structure, chemistry, and antimalarial properties of mefloquine-aziridine. Heterocycles. 15, 925-933 (1981).

2. Bertz, S. H., Adams, W. O. and Silverton, J. V. 2-5-Bis(methoxy-carbonyl)4-hydroxycyclopent-2-en-1-one as an intermediate in Weiss' glyoxal reaction. Analogous chemistry of malondialdehyde. J. Org. Chem. 46, 2828-2830 (1981).

3. Mitschka,. R., Oehldrich, R., Takahashi, K., Cook, J. M., Weiss, U. and Silverton, J. V. General approach for the synthesis of polyquinanes. Facile generation of molecular complexity via reaction of 1,2-dicarbonyl compound with dimethyl 3-ketoglutarate. Tetrahedron, 37, 4521-4542 (1981).

4. Mauger, A. B., Stuart, O. A., Highet, R. J., and Silverton, J. V. Synthesis of an actinomycin-related peptide, cyclo-(Thr-D-Val-Pro-Sar-MeAla), and conformational studies by nuclear magnetic resonance and X-ray crystallography. J. Amer. Chem. Soc., 104, 174-180 (1982).

5. Han, W. C., Takahashi, K., Cook, J. M., Weiss, U. and Silverton, J. V. Regiospecific cleavage of strained tri- and tetraquinane beta-diketones via retro-Claisen reaction. J. Amer. Chem. Soc., 104, 318-321 (1982).

6. Silverton, J. V., Limn, W., and Miles, H. T. 2-Amino-8-methyladenosine 5' monophosphate dihydrate. A nucleotide with C4'-exo conformation and "Triple-stranded" packing. J. Amer. Chem. Soc., 104, 1081-1087 (1982).

7. Sayer, J. M., Yagi, H., Silverton, J. V., Friedman, S. L., Whalen, D. L. and Jerina, D. M. Conformational effects in the hydrolyses of rigid benzylic epoxides. Implications for diol epoxides of polycyclic hydrocarbons. J. Amer. Chem., Soc., 104, 1972-1978 (1982).

8. Nishio, N., Blum, M. S., Silverton, J. V. and Highet, R. J. Structure of humistratin: a novel cardenolide from the sandhill milkweed Asclepias humistrata. J. Org. Chem., 47, 2154-2157 (1982).

9. Silverton, J. V., and Akiyama, T. The structure of gardneramultine. J. Chem. Soc., in press (1982).

10. Pandey, R., Misra, R., and Silverton, J. V. The structure of fredricamycin. J. Amer. Chem. Soc., in press (1982).

11. Takahashi, K., Jacobson, A. E., Mak, C-P., Witkop, B., Brossi, A., Albuquerque, E. X., Warnick, J. E., Maleque, M. A., Bavoso, A., and Silverton, J. V. 2-Desamylperhydrohistrionicotoxin. J. Med. Chem., in press (1982).

12. Buchs., P., Rice, K. C., Brossi, A., Silverton, J. V. and Potenzone, R. Spectroscopic, optical and crystallographic properties of cis-S-6'-bromo-N-formylnorreticulene. J. Org. Chem., in press (1982).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01006-11 CH

PERIOD COVERED

October 1, 1981 - September 30, 1982

TITLE OF PROJECT (80 characters or less)

The Characterization of Natural Materials

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. J. Highet	Research Chemist	CH	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemistry

SECTION

Structural Nuclear Magnetic Resonance Section

INSTITUTE AND LOCATION

NHLBI-NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

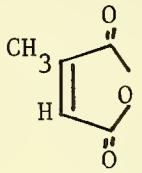
SUMMARY OF WORK (200 words or less - underline keywords)

1. J-modulated ^{13}C spectra. An experiment has been devised to allow the determination of vicinal C-H couplings on older spectrometers.

Streptomyces metabolites. The stereochemistry of the sugar moiety of the chrysomycin series has been established.

PHS-6040
(Rev. 2-81)

1. J-modulated ^{13} Spectra. An experiment has been devised to permit the characterization of trisubstituted double bonds by ^{13}C spectra modulated by vicinal C-H couplings. Following a 90° pulse at ^{13}C frequencies, the nuclear precessions are allowed to develop with proton coupling for a period of $1/2 \cdot {}^{3}J_{CH}$-typically 30 to 80 msec. Proton decoupling is now turned on and the free induction decay collected. Fourier transform produces a spectrum with the relevant signal nulled. The method allows the identification of critical couplings with resolution of noise-decoupled spectra on older spectrometers not capable of two-dimensional spectra. It has been demonstrated on citraconic anhydride, 1, and dimethyl acrylic acid, 2.



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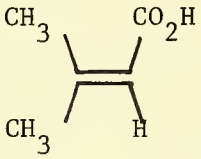

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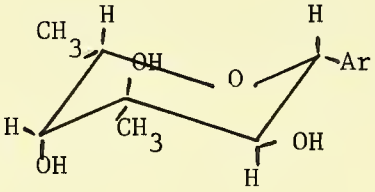

3

2. Streptomyces metabolites. In collaboration with U. Weiss, NIAMDK, the stereochemistry of the sugar moiety of the chrysomycin series has been established by studies of the intramolecular nuclear Overhauser effects. Irradiation of the 5'-H enhances the H-1' signal, demonstrating their cis-diaxial positions. Irradiation of the 7'-methyl has little effect on H-1' and H-5', eliminating an axial position for the 7'-methyl. Along with considerations of the observed couplings, the observations show the sugar to be 6-deoxy-7-methylgulose, 3, or its mirror image.

3. Incidental studies.

(a) (In collaboration with J. W. Wheeler, Howard Univ.), Double irradiation experiments on the hyena lactone 224 have located the double bond, showing the molecule to be the lactone of 14-hydroxy-12-tetradecenoic acid.

(b) (In collaboration with K. Rice and A. Brossi, NIAMDK). A survey of the ^{13}C spectra of compounds related to 6'-bromo-N-formyl nor-reticuline has revealed no effects of restricted rotation other than those associated with the formamide.

Publications

1. Khetrapal, C. L., and Highet, R. J. ^{1}H NMR spectra of partially oriented 1-propynlbenzene. Org. Mag. Res. 16: 117-118, 1981.

2. Pohl, L. R., Branchflower, R. V., Highet, R. J., Martin, J. L., Nunn, D. S. Monks, T. J., George, J. W., and Hinson, J. A. The formation of di-glutathionyldithiocarbonate as a metabolite of chloroform, bromotri-chloromethane, and carbon tetrachloride. Drug Metab. and Disp. 9: 334-339, 1981.

3. Mazzochi, P. H., Khachik, F., Wilson, P., Houk, K. N., Rondan, N., and Highet, R. J. Phytochemical additions of alkenes to N-methylphthalimides--The effect of aryl substituents on the direction of addition. J. Amer. Chem. Soc., 103: 6498-6499, 1981.

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6. Monks, T. J., Pohl, L. R., Gillette, J. R., Hong, M., Highet, R. J., Ferretti, J. A., and Hinson, J. A. Stereoselective formation of bromo-benzene glutathione conjugates. Chem. Bio. Interactions., in press.

7. Nishio, S., Blum, M. S., Silverton, J. V., and Highet, R. J. Structure of humistratin: A novel cardenolide from the sandhill milkweed Asclepias humistrata. J. Org. Chem. 47: 2154-2157, 1982.

8. Blum, M. S., Jones, T. H., Snelling, R. R., Overal, W. L., Fales, H. M., and Highet, R. J. Systemetic implications of the exocrine chemistry of some hypoclinea species. Biochem. Syst. Ecol, in press.

9. Tokuyama, T., Yamamoto, Y., Daly, J. W., and Highet, R. J. Histrionico-toxins. Carbon-13 magnetic resonance spectral assignments and structural definitions of further alkaloids from poison frogs (Dendrobatid). Tetrahedron, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01012-09 CH

PERIOD COVERED
October 1, 1981, to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Peptide Biochemistry

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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2.5	2.5	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A new mastoparan-like peptide has been isolated from yellow jacket (Vespula maculifrons) venom. Immunoreactive mastoparan has been observed in rat brain and human urine. Mastoparan binds strongly to mammalian membranes. Immunoreactive ranatensin has been detected in rat brain by immunocytochemistry and by radioimmunoassay. Intraventricularly injected ranatensin delays the onset of morphine or met-enkephalin catatonia in the rat. The stimulant in wasp (Polistes annularis) venon of the adenylate cyclase of neuroblastoma-glioma hybrid cell membranes has been isolated. It is adenosine, a previously known stimulant. More Gila monster peptide has been isolated and the end-groups identified. There is a high correlation (and some notable exceptions) with the ability of polypeptides to lyse liposomes and release histamine from rat mast cells.

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Objectives:

1. Isolate, characterize, and determine the significance of new biologically active peptides from natural sources.

2. Develop more effective methods for the microanalysis of peptides.

Methods: High-performance liquid chromatography (HPLC) and other chromatographic techniques, bioassay, antibody production and evaluation.

Major Findings:

Since simple screening tests are vital for the discovery of new peptides, we have compared 41 polypeptides for their ability to lyse liposomes and release histamine from rat peritoneal mast cells. There was a high correlation between the tests but there are notable exceptions. Most active in both tests were bee venom, mastoparan, polistes mastoparan, melittin and gramicidin S. Intermediate activity was observed in the liposome tests with apamin, compound 48/80 eledoisin-related peptide, granuliberin R, polymyxin B, somatostatin, and substance P, but with mast cells, this group was as active as the first group. Interestingly, polisteskinin and calcium ionophore were inactive on liposomes but were about as active as the above peptides in causing histamine release from mast cells.

The liposome lysis test was used to assay chromatographic fractions of yellow jacket (Vespula maculifrons) venom extracts. Two active fractions have been isolated. The amino acid composition of one is Gly_2, Val_1, Ile_1, Leu_5 Phe_1, Lys_2. The peptide has not yet been analyzed for proline. In addition to the second peak in Vespula maculifrons, another peak of activity has been observed in hornet (Dolicovespula maculata) venom.

Procedures have been set up in the laboratory to do amino acid and end-group analyses. The latter are performed primarily by the dansyl procedure using micro-TLC and HPLC for the identification of the dansyl amino acids. Carboxypeptidases A, B, and Y also have been successfully employed. We have found that Hageman factor inhibitor that we isolated from pumpkin seeds (Z01 HL 01018-23CH)) contains N-terminal arginine and C-terminal glycine.

More Gila monster peptide has been isolated. Preliminary analysis of the amino acid composition indicates: Asp_1, Thr_2, Ser_{4-5}, Glu_3, Pro_3, Gly_1, Ala_3, Ile_1, Leu_{4-5}, Tyr_{0-1}, Phe_1, His_1, Lys_{2-3}, Arg_1. End-group analysis indicates N-terminal histidine and C-terminal serine.

The titer of antibody to ranatensin (and the antibody to bombesin) has been increased. This antibody which recognizes neither bombesin nor GRP has been used in the discovery of immunoreactive ranatensin in rat brain. The peptide elutes identically to ranatensin on Sepadex G-25 columns but is resolved from ranatensin into three immunoreactive peaks by HPLC. Interestingly, one peak, but not the other two is recognized by an antibody specific for bombesin. This peak, therefore, has both ranatensin and bombesin antigenic determinants but is identical to neither.

Antibody has been used to visualize ranatensin in neuronal processes by immunocytochemistry in certain regions throughout the brain and the distribution determined by RIA. The distribution is different from that described for bombesin, a related peptide.

In previous experiments we found that met-enkephalin shifts the dose-response curve of ranatensin making it less potent on the guinea pig ileum. We have now found the converse. When morphine (10 μg) or met-enkephalin (100μg) are injected intraventricularly, strong catatonia appears in 2-3 min. However, when 20 μg of ranatensin was simultaneously injected, catatonia was delayed and did not appear until 10-12 min. The closely related bombesin does not share this morphine-antagonistic property of ranatensin.

Mastoparan is a tetradecapeptide (isolated from wasp venom) noted for its ability to degranulate rat peritoneal mast cells. We have undertaken a study to determine if it, or a related peptide, occurs in higher animals. Antibodies have been prepared against mastoparan and a radioimmunoassay developed using [^{125}I]Tyr-mastoparan. Immunoreactive mastoparan has been detected in rat brain and human urine. Two peaks of immunoreactivity are seen on Sephadex G-25. The first peak elutes with proteins and does not give a line parallel to the standard. The second peak elutes in the peptide fraction and gives a parallel line. The peptide peak is equivalent to 0.5 ng mastoparan/ml. Less is known about the brain immunoreactivity. Several regions contain about 0.4 ng/g but the hypothalamus contains 0.8 ng/g.

^{125}I-Tyr-mastoparan also has been used to test for binding protein(s) or receptors in rat brain membranes. The labeled material was purified by HPLC and the diiodinated peptide used. Several methodological problems, relating to the highly adsorptive nature of the peptide, had to be overcome. The final procedure employs Whatman GF/C filters presoaked in 0.1% poly-L-lysine. Binding to rat brain membranes is linear in the range 50-250 μg protein, specific, and saturable. Of numerous peptides tested, only structurally related peptides, melittin and apamin (polypeptides from bee venom) displaced [^{125}I]-Tyr-mastoparan. Binding was also inhibited by mono- and divalent cations, the latter being more potent. Bound peptide was largely recoverd unaltered from the membrane fraction. Somewhat unexpectedly, [^{125}I]-Tyr-mastoparan binding was similar with membrane fractions from various brain regions and membrane fractions from various organs. Thus, it appears that binding is not to a specific receptor, but to some structure common to all membranes. Since melittin, a well-known membrane-active polypeptide, displaces mastoparan and is known to stimulate phospholipase A_2, a constituent of membranes, the possibility that mastoparan interacts with phospholipase should be considered.

Neuroblastoma-glioma hybrid cell membranes contain a well-characterized receptor for different neuromodulators including met-enkephalin, endorphin, acetylcholine, noradrenaline, adenosine and prostaglandins. Stimulation of these receptors causes an inhibition or stimulation of adenylate cyclase with parallel changes in the production of cAMP. Extracts of 1) Vespula pennsylvanica, 2) Vespula germanica, 3) Vespula maculifrons, 4) Polistes annularis, 5) Dolichovespula maculata 6) Vespa crabro and 7) Gila monster venom were tested. Venoms 1-4 stimulated the cyclase whereas 5,6 and Gila monster were inactive. Venom 4

had the strongest excitatory activity. The active material was isolated and identified as adenoside, a well-known stimulant.

Proposed Course:

1) To set up micro-sequencing facility and new peptide screening tests

2) To sequence the Gila monster peptide and the peptide form Vespula maculifrons

3) To characterize immunoreactive ranatensin and mastoparan in rat brain and mastoparan in human urine

4) To study the consequences of mastoparan binding to membranes

5) To discover new peptides

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01016-12 CH

PERIOD COVERED

October 1, 1981, to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Clinical Biochemistry of the Kallikrein-Kinin System

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1) In the rat kidney immunoreactive kallikrein content was highest in the outer cortex and decreased progressively toward the papillary tip. In microdissected cortical nephron segments, the highest kallikrein content was found in the connecting tubule and in immediately adjacent segments. 2) Specific binding of bradykinin to membrane preparations from homogenized cortex was higher than medulla. In microdissected nephron segments, significant binding was found in the length from the thick ascending limb to the collecting tubule. 3) "Urine" produced by the isolated perfused rat kidney contains kinin and kininogen as well as kallikrein. 4) While the immunoreactive kallikrein levels of rat renal lymph appear to be similar to that in thoracic lymph, renin levels are 400-fold those of thoracic lymph. In contrast renal lymph contains about one-tenth as much angiotensin converting enzyme as either plasma or thoracic lymph. Paradoxically, angiotensin I could not be detected in thoracic lymph whereas renal lymph contained significant levels. Renal lymph also contains kinin and kininogen.

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PHS-6040
(Rev. 2-81)

Objectives: To establish the role of the kallikrein-kinin system in the kidney.

Methods: Microdissection of nephrons, collection of renal and thoracic lymph, bioassay, radioimmunoassay, radiochemical and chromogenic substrate assays, chromatographic and electrophoretic techniques, immunochemical procedures.

Major Findings:

Distribution of Immunoreactive Kallikrein Along the Rat Nephron: The kallikrein-kinin system is believed to play an important role in the regulation of renal tubular water and electrolyte transport. To obtain more convincing evidence, we have studied the distribution of cellular kallikrein along the nephron. Using a direct radioimmunoassay, we have localized kallikrein along the cortico-medullary axis of the rat kidney and in microdissected rat cortical nephron segments. Kallikrein content was highest in the outer cortex and decreased progressively toward the papillary tip. The kallikrein content in the cortex was significantly increased following dietary sodium restriction. In microdissected cortical nephron segments, the highest kallikrein content was found in the connecting tubule (155±31 (S.E.) pg/mm tubule length (n-5). In addition, a significant amount of kallikrein was found in distal convoluted tubules (50±7 pg/mm, n=6), initial collecting tubules (74± 21 pg/mm, n=6), and cortical collecting ducts (37±8 pg/mm, n=5). Little or no kallikrein was found in glomeruli, proximal convoluted tubules, proximal straight tubules, and cortical thick ascending limbs. These results are compatible with the site of action of the kallikrein-kinin system in the cortical segments of the rat distal nephron.

The Kallikrein-Kinin System in Rat Renal Lymph: We have extended our previous studies (Z01 HL 01016-11C) on renal lymph to include a comparison with thoracic lymph. Renal lymph contains 260± 12 ng/ml ($\bar{X}$ ± SEM, N-14) of immunoreactive glandular kallikrein. Like the immunoreactive glandular kallikrein in plasma, it is biologically inactive and 35,000 dalton and 85,000 dalton peaks are seen on gel filtration. While the kallikrein level in renal lymph appear to be similar to that in thoracic lymph (kallikrein assay of thoracic lymph are not parallel to the standard, decrease on freezing and thawing, and show a different profile on gel filtration) renin levels (280 ng AI/ml/hr) are 400-fold those of thoracic lymph and 50-fold those of plasma. In contrast, renal lymph contains approximately one-tenth as much angiotension converting enzyme (370 units/ml) as either plasma on thoracic lymph. Paradoxically renal lymph contains 125 ng/ml AI and thoracic lymph undetectable levels. It appears that while renin is elaborated into the circulation by the kidney, this organ is not a major source of the immunoreactive glandular kallikrein in plasma. Our results so far do not, however, allow us to exclude the possibility that part of the immunoreactive kallikrein in lymph orginated from the tubular cells and was initially biologically active. Renal lymph also contains kinin (20 ng/ml) and kininogen. The latter was determined by incubation of lymph with kallikrein; 110 ng/ml of kinin was liberated.

Kinin Receptors in the Kidney: To gain a better understanding of the role of the kallikrein-kinin system in the kidney, it is necessary to know which components (kallikrein, kininogen, kinins, kininases) occur in this organ, their distribution, their site of interaction and the localization of kinin receptors. Initial studies on kinin receptors have shown that freezing of membrane fractions does not significantly alter specific binding of [^{3}H] -bradykinin. This information indicated the feasibility ofextending the study to segments of microdissected nephrons where three days are required to obtain enough material for study. Daily collections were stored at -80° until tested (usually on day four). Specific binding of [^{3}H] bradykinin to membrane preparations from homogenized cortex and medulla was 5 and 43 fmol/mg protein, respectively. Microdissected nephron segments examined were proximal convoluted tubule and the length from the thick ascending limb of Henle's loop to the collecting tubule. No specific binding was detected in the proximal convoluted tubule but 32 fmol/mg was found in the length from the thick ascending limb to the collecting tubule.

The Kallikrein-Kinin System in the Isolated Perfused Rat Kidney: The isolated perfused kidney is an useful model to study the role of the kallikrein-kinin system in this organ. It was previously known that this preparation elaborates kallikrein into the perfusate and into the "urine". We have extended these studies to an analysis of the "urine" for kinin and kininogen to determine if all the necessary components are present and active. The preparation clears inulin normally and does not show any evidence of tubular damage (ALK phosphatase, LDH tests are negative) during the first hour. In the periods 10-30 min and 30-60 min the urine contained 1.4 $\pm$ 0.3 and 0.5 $\pm$ 0.1 g($\bar{X}$ $\pm$ S.D., n-4) of immunoreactive kallikrein, respectively, 0.3 $\pm$ 0.1 and 0.04$\pm$ 0.08 ng kinin; and 1.1$\pm$ 0.4 and 0.7 $\pm$ 0.1 ng additional kinin released after incubation with trypsin. This additional kinin evidently comes from kininogen. Only one third as much kallikrein is produced if the kidney is pretreated with puromycin; kinin and kininogen values do not change.

Other Studies: Kallikrein in human urine occurs as both the proenzyme and the free, active enzyme in roughly equal amounts. Studies on urine concentrates (unconcentrated urine has not been tested) indicate that part of the kallikrein is also bound to another protein(s) and can be readily separated from kallikrein on a Sephadex G-150 column. Roughly one-third of the total trysin activatable esterase is complexed. It is not immunoreactive and about 15% of the esterase is active before trypsin treatment. The complexed kallikrein was about two-thirds dissociated when chromatographed on a Sephacryl S-200 SF column with 1M salt. That two-thirds eluted where standard kallikrein elutes, it was immunoreactive and contained only active esterase (i.e. trypsin did not cause an increase in esterase activity). The remaining one-third apparently still behaves like complexed kallikrein as it eluted earlier from the column, was not immunoreactive and esterase activity increased substantially after trypsin treatment.

Previous studies on stored frozen urine from patients who had their thyroid glands removed because of aldosterone-producing adenomas indicated low levels of kallikrein and virtually all of it was in the form activatable by trypsin (prokallikrein). Subsequent studies on fresh urine, however, revealed normal levels of active kallikrein and prokallikrein. Thyroidectomized rats also appear to have normal levels.

Urine from one monkey was tested for components of the system. Results were similar to human urine with the exception that the RIAs for kallikrein and HMW kininogen gave lines which were not parallel to the standards.

Proposed Course: To localize and characterize kinin receptors in the kidney. To compare fresh and frozen samples of rat renal lymph, thoracic lymph, and plasma for the levels of immunoreactive kallikrein, renin, angiotensin converting enzyme, and angiotensins I and II. To compare the gel filtration profiles of immunoreactive kallikrein in the lymph and plasma samples. To study the production of kallikrein, kininogen and kinin in the isolated perfused rat kidney, and to ascertain determinants and consequences of kinin formation and destruction in this preparation.

Publications:

1. Proud, D., Perkins, M., Pierce, J. V., Yates, K. N., Highet, P. F., Herring, P. L., Mangkornkanok/Mark, M., Bahu, R., Carone, F., and Pisano, J. J. Characterization and localization of human renal kininogen. J. Biol. Chem. 256: 10634-10639, 1981.

2. Marks, E., Hojima, Y., Frech, M. E., Keiser, H., and Pisano, J. J. An inhibitor from corn blocks the hypotensive action of plasma protein fraction and active Hageman factor. Thrombosis Research 23: 97-102, 1981.

3. Horwitz, D., Proud, D., Lawton, W. J., Yates, K. N., Highet, P., Pisano, J. J., and Keiser, H. R. Effects of restriction of sodium or administration of fludrocortisone on parotid salivary kallikrein in man. J. Lab. Clin. Med. 99: 0000, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01018-25 CH

PERIOD COVERED

October 1, 1981, to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Biochemistry of the Kallikrein-Kininogen-Kinin System

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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1.2	1.2	

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SUMMARY OF WORK (200 words or less - underline keywords)

Human renal kallikrein with a specific activity 2.5 times that found for hollow fiber concentrates of urine can be recovered in about 95% recovery by DEAE-cellulose batch adsorption of 120-liter lots of urine. In preliminary studies with 40% pure renal prokallikrein no evidence of an activation peptide was seen by high performance liquid chromatography (HPLC) after activation by catalytic amounts of trypsin. Thin-lay chromatography (TLC) of a dansylated, then acid-hydrolyzed incubation mixture of prokallikrein and trypsin revealed fluorescent spots for dansyl (Dns)-ε-lysine, Dns-glutamic or -aspartic, and Dns-isoleucine, -leucine, or -valine.

Immunofixation of human plasma kininogens on electroblot transfers from alkaline polyacrylamide gels after electrophoresis of fresh samples of plasma revealed antigen patterns very similar to the stained protein band patterns previously obtained with kininogens purified by immunoaffinity chromatography

4/6

PHS-6040
(Rev. 2-81)

Objectives: Purification of glandular kallikreins and prokallikreins and of components of the plasma kinin, clotting, and fibrinolytic systems for purposes of characterization and of production of specific antiserums. Preparation of purified specific antibodies and isolation from plant and animal sources of specific inhibitors for human plasma and glandular proteases (kallikreins, Hageman factor, plasmin, thrombin, Factor XI, elastases, etc.) for biochemical, clinical, and other studies. Preparation of affinity adsorbents from purified antibodies, antigens, enzymes, and inhibitors for purification and other purposes, such as devising specific biochemical and radioimmunochemical assays. Application of these purified materials, affinity adsorbents, and assay methods to studies of normal and pathological states in man and other primates.

Major Findings:

1. Human Renal Kallikrein and Prokallikrein. a. Purification. DEAE-cellulose batch adsorption now replaces hollow fiber ultrafiltration (Z01 HL 01018-24 CH) as the first step in the isolation of kallikrein and prokallikrein from urine. The advantages of the new method over the previous one are: (1) the volume of urine processeed is limited only by such physical factors as the size of the container and a means for vigorously mixing the urine and adsorbent; (2) the specific activity is 2.5 times as large; (3) each lot of urine (120 liters at present) can be processed in 2 days instead of about 4 weeks for the Amicon H1P8 hollow fiber ultrafiltration; consequently, (4) the adsorption method can be done at room temperature instead of in the cold room; and (5) the recovery of prokallikrein should be greater. Thus far, 900 liters of urine have been concentrated by this method to 9 liters of NaCl eluate in about 95% recovery of esterase activity.

Following a recent paper reporting the hydrophobic chromatographic purification of hog urinary kallikrein with Octyl-Sepharose CL-4B, we did batch experiments with this adsorbent, as well as with Phenyl-Sepharose CL-4B, to test the feasibility of hydrophobic chromatography of human renal kallikrein and prokallikrein. Both adsorbents bound appreciable amounts of kallikrein from a hollow fiber urine concentrate and at least 95% of the starting esterase activity was accounted for in each experiment by the activity found in the supernatant solution and the water eluate. Phenyl-Sepharose had about twice the capacity for kallikrein as Octyl-Sepharose.

b. Chemical Studies. To test whether trypsin activation of renal prokallikrein releases an activation peptide or cleaves an internal bond, we have done several preliminary experiments on incubation mixtures of prokallikrein and trypsin. HPLC on Toya Soda TSK-G3000SW and TSK-LS-410 ODS columns of such mixtures in which all of the prokallikrein had been activated by catalytic amounts of trypsin gave no evidence of molecular weight or adsorptive changes, or of the appearance of an activation peptide. Dansylation of the incubation mixtures followed by acid hydrolysis and TLC showed no fluorescent spots (not even of Dns-ε-lysine) with the incubation of prokallikrein alone. However, fluorescent spots were observed with the incubation mixture of prokallikrein and trypsin: one spot corresponded to Dns-ε-lysine, another to Dns-glutamic or -aspartic, and the third to Dns-isoleucine, -leucine, or -valine (Isoleucine has been identified as the amino-terminal residue of naturally occurring human renal kallikrein).

2. Human Plasma Kininogen. Using the sheep antiserum to B2α low molecular weight (LMW) kininogen prepared in our laboratory (Z01 HL 01018-20 LC) and immunofixation by the peroxidase-antiperoxidase method of antigens on electroblot transfers from alkaline polyacrylamide gel electrophoretograms of freshly prepared human plasma samples, Dr. Azen consistently found three kininogen antigen bands in the transferrion region, as well as faint, diffuse bands in the γ-globulin region, when blood samples were collected in the presence of 0.01% Polybrene which prevents the surface activation of Factor XII. When Polybrene was not used, however, the slower-moving diffuse bands were sharper but the pattern was not the same from sample to sample. This is consistent with different degrees of limited proteolysis of high molecular weight kininogens by the plasma kallikrein produced by Factor XII_a. As expected for LMW kininogens, the three faster bands were unchanged since these kininogens are very poor substrates for plasma kallikrein. The antigen patterns were very similar to the stained protein bands seen previously in alkaline polyacrylamide gel electrophoretograms of kininogens purified by immunoaffinity chromatography (NHLI-284).

Proposed Course of Project: Human Renal Kallikrein and Prokallikrein. Methods of purifying prokallikrein further, separating it readily from kallikrein, and preventing its "spontaneous" conversion to kallikrein will be sought. Further experiments to test for the presence in urine and fractions from it of an activating enzyme of renal prokallikrein will be done. With more highly purified prokallikrein, as well as with a rapid method for resolving and quantifying kallikrein and prokallikrein, more definitive experiments can be done on the activation of prokallikrein.

Human Plasma Kininogen. Further immunofixation experiments will be done on the kininogens purified by immunoaffinity chromatography and on any available fresh plasma samples from persons with different kininogen deficiencies.

Publications:

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2. Lawton, W.J., Proud, D., Frech, M.E., Pierce, J.V., Keiser, H.R., and Pisano, J.J.: Characterization and origin of immunoreactive glandular kallikrein in rat plasma. J. Biochem. Pharmacol. 30: 1731-1737, 1981.

3. Proud, D., Perkins, M., Pierce, J.V., Yates, K.N., Highet, P.F., Herring, P.L., Mangkornkanok/Mark, M., Bahu, R., Carone, F., and Pisano, J.J.: Characterization and localization of human renal kininogen. J. Biol. Chem. 256: 10634-10639, 1981.

4. Ørstavik, T.B., Brandtzaeg, P., Nustad, K., and Pierce, J.V.: Effects of different tissue processing methods on the immunohistochemical localization of kallikrein in the pancreas. J. Histochem. Cytochem. 29: 985-988, 1981.

Annual Report of the
Clinical Hematology Branch
National Heart, Lung, and Blood Institute
October 1, 1981 to September 30, 1982.

The research of this Branch is directed toward understanding the underlying causes and developing effecting treatment for the major red cell disorders. Red cell diseases which produce significant morbidity and mortality include thalassemia, severe hemoglobinopathies of which sickle cell anemia is the most common, and the various syndromes of bone marrow failure. In our laboratory investigations, we attempt to focus the most advanced techniques and conceptual knowledge on several problems. These include analysis of the differentiation and maturation of erythroid cells, elucidation of globin gene structure and expression, and investigation of the phenomenon of hemoglobin switching whereby one hemoglobin is replaced by another during normal development. Our clinical projects are mainly directed toward devising or applying new therapeutic strategies. Major conceptual developments have led to potentially effective therapy for all three major categories of disease, thalassemia, sickle cell anemia, and severe aplastic anemia. Hence, during the past year much of our effort has been re-directed toward refining and applying these therapeutic strategies to the treatment of patients with severe red cell disorders.

Patients with either severe β thalassemia or sickle cell anemia could benefit from increased production of fetal hemoglobin. During normal human development, Hb F ($\alpha_2\gamma_2$), produced in utero, is replaced during the perinatal period with Hb A ($\alpha_2\beta_2$). If both β globin genes are defective, either because of mutations which reduce the amount of functional mRNA which is produced or because of mutations which results in a structurally abnormal globin (e.g., the β^S globin), the perinatal switch in hemoglobin production leads to the onset of the disease. Reactivation of the γ globin gene in adults could ameliorate the severity of these conditions.

Recent advances in our knowledge about gene structure and function has provided a therapeutic approach to this problem. Actively expressed genes are generally hypo-methylated compared to DNA sequences which are unexpressed. The drug, 5-Azacytidine, both inhibits DNA methylation in replicating cells and also has been shown to activate a variety of genes in vitro. Recent data indicating that this drug activates the γ globin gene in adult baboons has prompted our therapeutic trial in humans.

Two patients have been treated to date. (See Individual Project Report: Effect of 5-Azacytidine on Fetal Hemoglobin Synthesis in Patients with Severe β Thalassemia and Sickle Cell Anemia). One individual with β thalassemia had a seven-fold increase in γ globin synthesis which temporarily corrected the abnormal α/non-α globin biosynthetic ratio characteristic of this condition. Reticulocytosis and an increase in hemoglobin concentration reflected the therapeutic benefit of increased Hb F production. Recently, a second patient who has sickle cell anemia has been treated. A several-fold increase in γ globin synthesis resulted in an increase in Hb F and complete disappearance of sickled red cells from the patient's peripheral blood.

These data establish that 5-Azacytidine is effective in increasing Hb F production. Our immediate objectives are several. First we hope to develop a regimen which is potentially useful or a chronic basis. An animal model will be developed in an effort to maximize the therapeutic benefit achieved by drug administration by varying dose, frequency of administration, and combining the

drug with other agents which perturb erythroid progenitor cell behavior. Finally, selected patients with advanced iron overload who are at immediate risk for death due to cardiac disease, will be treated repeatedly with 5-Azacytidine. Chelation therapy, shown in our studies to be relatively effective (see Individual Project: Iron Chelation in Transfusional Hemosiderosis), will be given in an effort to maximize iron excretion during the period of 5-azacytidine treatment.

Severe aplastic anemia, a nearly uniformly fatal condition has been shown to respond by ourselves and others to administration of anti-thymocyte globulin. (See Individual Project: Hematopoiesis in Bone Marrow Failure). An immunological basis for aplastic anemia has been suggested by previous observations although the precise mechanism of action of ATG has not been defined. This serum, produced by immunization of horses with human thymocytes, may have broad reactivity with many human cells. Current experiments are designed to define its specificity. Nonetheless, the activity of this agent in the treatment of aplastic anemia has been established prompting us to organize a multi-institutional trial designed to develop a regimen with maximal therapeutic benefit. Patients treated at NHLBI and in other institutions will be studied intensely in an effort to obtain insights into the pathogenesis of aplastic anemia and the mode of action of ATG.

Much effort in this laboratory is focused on the analysis of gene structure and function. A euykaryotic expression system has been developed utilizing a plasmid vector and a monkey kidney cell line (see Individual Project Report: Use of Viral Regulatory Sequences to Facilitate Gene Transfer and Analysis of Gene Function). Using this experimental approach, we have shown that the human γ and β globin gene promoters, but not the α globin gene promoter, require a remotely situated enhancer element for function. Furthermore, the hypofunction of the γ globin gene has been shown to be due to its relatively inactive promoter. Function of the β globin gene has been shown to require sequences between 80 and 127 nucleotides upstream from the site of initiation of transcription but sequences further upstream are not required, and furthermore may in fact inhibit promoter function.

Our efforts to characterize thalassemic globin genes have proceeded along two lines. (See Individual Project Report: Molecular Defect in β Thalassemia). A highly efficient technique for mapping the β globin gene transcripts in erythroid cells have been applied to the study of 15 patients. Two defects have been shown to occur with high frequency, one involving abnormal splicing of sequences transcribed from intron 1 of the human β globin gene and the second reflecting generally retarded removal of intervening sequence RNA. Molecular cloning has yielded two thalassemic globin genes which have been extensively characterized. One new mutation has been defined. A single nucleotide substitution in codon 24 of the human β globin gene activates a cryptic splice site which generally retards RNA processing and decreases production of functional β globin mRNA. This novel mutation illustrates the unique stringent sequence requirements for effective RNA splicing since an otherwise silent substitution in the coding portion of this gene results in severe disease. The second thalassemia gene studied is affected by a single nucleotide substitution which generates a premature stop codon in the reading frame of β globin mRNA. This mutation, previously described by others, has been shown in our experiments to lead directly to a quantitative reduction in total β mRNA.

The human dihydrofolate reductase gene has been selected as an example of a constitutively expressed gene which encodes for a protein product essential for cell replication. (See Individual Project: Characterization of the Genes for Human Dihydrofolate Reductase in Normal Cells and in Cells Resistant to Methotrexate). Our intent is to compare the structure, and specifically the transcriptional signals, to the corresponding portions of the highly specilized globin genes. To date,

one half of the human dihydrofolate reductase gene has been isolated and sequenced. Additional experiments are in progress to obtain the remainder of the gene. One problem, evident from our effort to clone the dihydrofolate reductase gene, is the high probability, in such experiments of isolating non-functional pseudogenes which appear to be very frequent in the human genome. Currently, we are devising a strategy which depends on gene expression during all steps of gene purification. (See Individual Project Report: Development of a General Method for Gene Cloning Using Eukaryotic Expression Vectors). Such a cloning strategy should yield only functional genes and would markedly facilitate the effort to obtain the genes which encode for functionally important but quantitatively insignificant proteins. Among these are cellular receptors for growth factors such as erythropoietin, insulin and transferrin.

Much of human DNA is composed of moderately repetitive DNA sequences. We have characterized two long interspersed repetitive DNA sequence families. One has roughly 4000 members, it is dispersed throughout the entire genome, and lacks obvious function. (See Individual Project Report: Characterization of a Repeat DNA Sequence Family). The second family has many fewer members, perhaps as few as 3-5. (See Individual Project Report: Characterization of the Genes for Human Dihydrofolate Reductase). These DNA sequences are particularly interesting because of their origin. Our efforts to clone the human dihydrofolate reductase gene has yielded two intronless DHFR genes. They have been extensively studied and shown to be derived from processed RNA intermediates which are converted into DNA and reinserted into the genome. One of these intronless genes is of very recent evolutionary origin and may prove useful in defining the mechanism by which these genes arise.

Another major area of experimental effort in the laboratory is the study of hematopoiesis. During the past year, data has been obtained implicating a human parvovirus in the pathogenesis of transient erythroblastopenia. This bone marrow failure syndrome apparently occurs because of the unique suceptibility of the human erythroid progenitors to infection by this virus. This observation provides clear cut evidence that viruses can cause bone marrow hypofunction and provides impetus to search for viral agents which may be involved in severe aplastic anemia.

A major problem inherent in the study of hematopoietic stem cells, is the fact that these cells are present in very low number, and furthermore the features which make these cells interesting--namely receptors for various growth factors--are present in very low concentration. To overcome these limitations, monoclonal antibodies have been derived which are directed against membrane components present on hematopoietic stem and progenitor cells. (See Individual Project Report: Use of Hybridoma Technology in the Study of Erythroid Differentiation). Two of these have been extensively characterized. The antigens with which these antibodies react clearly decrease in density during myeloid differentiation. One antibody has been shown, in preliminary experiments, to stimulate proliferation and colony formation by human K562 cells. This antibody also fails to react with a retinoic acid insensitive K562 line. These two observations raise the intriguing possibility that this antibody reacts with a growth factor receptor. Our plan is to characterize this protein in detail and potentially clone its gene by looking for its expression in transformed cells.

Earlier studies in this laboratory have been directed toward analysis of hemoglobin switching in the sheep experimental model. These efforts have been redirected for several reasons. First, the organization of the globin genes in sheep is quite different than that in man. Second, sheep lack a F-cell population which is characteristically present in man and other primate species. This residual

capacity to produce modern amounts of Hb F in a small fraction of bone marrow erythroid cells may be crucially important for activation of fetal hemoglobin production. Third, the demonstrated activity of 5-Azacytidine in stimulating fetal hemoglobin production in man eliminates at present, the need for the sheep model to explore methods for manipulation of hemoglobin synthesis. Rather, a primate model may be more important to define the mechanism of action of this drug and for devising the most effective therapuetic regimen.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02203 10 CHB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Molecular Defects in β Thalassemia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	Merrill Goldsmith	Staff Fellow	CHB NHLBI
Other -	Nick Anagnou	Visiting Scientist	CHB NHLBI
	Guglielmina Pepe	Visiting Scientist	CHB NHLBI
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COOPERATING UNITS (if any)

None

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

These studies are designed to define the molecular lesions affecting β globin production in patients with homozygous β thalassemia. Earlier work performed in this and other laboratories have suggested that β thalassemia is frequently caused by mutations which affect the normal pattern of processing of β globin mRNA precursors. During the past year, we have attempted to define defects in RNA processing which occur in bone marrow cells of patients with β thalassemia, and to clone genes from patients who have been identified as having novel RNA processing defects. Three types of abnormal RNA molecules have been demonstrated: the first two reflect defects which lead to abnormal processing of the smaller intervening sequence of the β globin gene transcript. The third is present in a patient who preferentially uses an upstream initiation site of RNA transcription which is infrequently used in normal bone marrow cells. Two thalassemic globin genes have been cloned, sequenced, and studied in a eukaryotic expression system. One of these genes contains a single base pair mutation in the first coding block of the β gene, resulting in an abnormal splicing pattern. The second gene contains a previously described mutation which prematurely stops translation of processed β mRNA. Transcripts from both of these abnormal genes appear to be relatively unstable in a eukaryotic expression system.

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PHS-6040
(Rev. 2-81)

Objective:

The objective of these studies is to use the naturally occurring model of defective gene expression provided by β thalassemia in order to understand gene regulation. Recognition of molecular lesions may provide insight regarding the specific mechanism of regulation of the expression of individual genes in human cells. Appreciation of the molecular basis of this disorder may suggest an approach for specific types of therapy.

Methods:

Mapping of globin mRNA transcripts:

1. Bone marrow RNA and DNA is purified by lysing bone marrow cells with guanidinium HCl and centrifugation in cesium chloride gradients. Purified RNA is hybridized to a single stranded globin DNA probe. The duplexes are exposed to S_1 nuclease, and protected DNA fragments are identified by size fractionation on polyacrylamide gels following denaturation, using an adaptation of the procedure of Berk and Sharp.

2. Preparation of single stranded globin gene probes: Various portions of the normal human α, β, γ, δ, and ε globin genes have been subcloned into bacteriophage, M13. This phage secretes one of its two strands into the culture medium; this single stranded recombinant globin -M13 DNA then serves as a template for synthesis of uniformly labeled single stranded probes. These probes are used to characterize and map globin mRNA precursors by the method of Berk and Sharp.

3. Cloning of thalassemic globin genes; the two globin genes that we have completely characterized were isolated from libraries of cloned DNA fragments. The bacteriophage library is amplified in a bacterial strain which harbors the vector, πVX, a small plasmid containing a segment of the β globin gene. During amplification, recombination occurs so that the πVX plasmid is inserted into recombinants that contains sequences homologous to the globin gene fragment. Since πVX also contains a suppressor tRNA gene, phage clones which acquire this plasmid by recombination, are also able to form plaques on a bacterial strain which lacks suppressor tRNA. This occurs because Charon 4A has amber mutations in genes for critical structural proteins. In addition, we have recently acquired a new strain of bacteriophage , Charon 21A. We are able to ligate Hind III cut genomic DNA directly into the "phosphatased" Hind III sites of this bacteriophage; complete digestion of genomic DNA with Hind III yields the intact β globin gene in a 7.5 kb Hind III fragment. This strategy enhances the frequency of cloning complete β globin genes. Combined with the πVX system, these two new methods should greatly facilitate the molecular cloning of new and interesting thalassemic genes.

Major Findings

1. Abnormal processing of intervening sequence 1 leads to β thalassemia: We have completely mapped the β globin gene transcripts from 15 patients homozygous for severe β thalassemia. RNA from 7 of these patients contained an abnormally processed RNA species which retains 19 nucleotides from the 3' end of IVS-I. This RNA processing abnormality is caused by a single nucleotide substitution which creates an alternate 3' splice in intervening sequence 1. In addition, nine of the 15 patients had an RNA processing abnormality which resulted in retarded removal of sequences transcribed from intron 1. At least 2 of these patients were shown to have a mutation at the 5' end of IVS1 which competely ablates the function of this splice donor site.

2. These studies have defined a previously unidentified upstream start site for β globin transcription. Although the significance of this upstream start is not yet clear, at least one thalassemic patient uses this start site preferentially. This patient probably has mutations in the sequences required for efficient initiation of transcription at the normal site.

3. Molecular cloning and characterization of β thalassemic globin genes: Two thalassemic genes were successfully cloned and characterized. The first of these contains a heretofore undescribed mutation, a single nucleotide substitution (T -> A) in codon 24. This substitution activates a cryptic splice donor site, and results in abnormal processing of RNA transcripts. Study of RNA transcripts generated from this gene in a eukaryotic expression system has shown these transcripts to be unstable. The second cloned gene containes a premature stop signal for translation in codon 39. This abnormality has been previously described, but we have demonstrated these transcripts to be unstable in our eukaryotic expression system, a previously undescribed finding which suggests that instability of these transcripts may account for the low levels of β mRNA observed in bone marrow cells.

Significance to Biomedical Research and the Institute Program:

Homozygous β thalassemia is a disease which causes severe morbidity and mortality. An understanding of the genetic basis of this disorder may add insight to the mechanism of gene regulation and may provide a basis for new strategies to alter gene function.

Proposed Course of Project

We will continue to map RNA transcripts acquired from bone marrow cells of thalassemic patients, hoping to find evidence of new types of mutations causing thalassemia. Many mutations causing abnormal RNA splicing have already been identified, and we have defined the *in vivo* consequences of two of these mutations. However, mutations affecting regulatory regions flanking the β globin gene have not yet been well characterized. One good candidate for such a mutation is a patient who utilizes an upstream transcription site preferentially. We will attempt to clone, sequence, and characterize the gene which causes this mutation. We will contiue our efforts to identify patients with regulatory mutations by studying RNA transcripts in patients with β^+ thalassemia; if patients of this type do not contain processing errors, they may be likely to have mutations affecting the regulatory regions.

Publications:

1. Ley, T.J., Anagnou, N.P., Pepe, G., and Nienhuis, A.w. RNA processing errors in patients with β thalassemia. Proc. Natl. Acad. Sci. USA, in press. August, 1982.
2. Nienhuis, A.W., Ketschmer, P., Kaufman, R., Kantor, J., Coon, H., and Goldsmith, M. Structure and Expression of Globin Genes. In "Advances in Red Cell Biology," Weatherall, D.J., Fiorelli, G., and Gorini, S. eds. Raven Press, New York, 1982. pp. 165-176.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02208 08 CHB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Iron Chelation in Transfusional Hemosiderosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI --	A. W. Nienhuis	Branch Chief	CHB NHLBI
	Patricia Griffith	Clinical Nurse Specialist	CHB NHLBI
Other -	T. Ley	Clinical Associate	CHB NHLBI
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LAB/BRANCH
Clinical Hematology Branch

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National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	

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☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

These studies are designed to evaluate the clinical benefits achieved by iron chelation in patients with chronic iron overload. Desferrioxamine is administered by subcutaneous infusion and iron removal is determined by quantitation of urinary iron excretion and careful recording of the total iron administration by transfusion. Those patients who have no evidence of cardiac disease are randomized to receive either ascorbic acid (3 mg/kg) or placebo. Sixty-five patients are now included in our long term chelation trial and of these 49 have been randomized to the ascorbic acid study. Most patients have now been followed for a minimum of three and many for four years. Currently all patients are undergoing an annual evaluation with the expectation that we will analyze all data in an effort to judge the clinical efficacy of chelation and the influence of ascorbic acid therapy.

PHS-6040
(Rev. 2-81)

Objectives:

The objectives of these studies are to evaluate available iron chelators to maximize their effectiveness, and to test new chelators as they become available. An effort is being made to develop clinical criteria which will be helpful to determine the efficacy of chronic chelation therapy. A randomized trial of supplemental ascorbic acid has been initiated to assess the value and/or toxicity of this agent in promoting mobilization of iron by desferrioxamine in patients with iron overload.

Methods:

Patient populations which participate in these studies include: 1) patients with transfusion dependent congenital or acquired anemia who require regular blood transfusions to sustain life, and 2) patients with idiopathic hemochromatosis at various stages in the process of iron removal by phlebotomy.

Clinical evaluation of organ function include the following:

1) Heart: An estimate of cardiac size by chest X-ray and electrocardiographic analysis is obtained. Echocardiographic studies are obtained to determine anatomical dimensions of the left ventricle and left ventricular function is assessed by resting ejection fraction. This year, follow-up radionucleotide angiograms with exercise are being performed on 15 patients who have been on Desferal for a period of 3-4 years.

2) Endocrine evaluation includes specific testing of the pituitary, thyroid, adrenal, pancreatic islets, and gonad function by baseline measurements and various provocative tests.

3) Liver function is determined by standard clinical testing. In addition, liver biopsies are performed to assess histology and to quantitate liver iron concentration.

4) Serial serum ferritin measurements are obtained to assess the utility of this parameter in estimating total body iron stores and also to follow the course of iron removal.

Major Findings:

No major new observations have proceeded from our study during the past 12 month follow-up period. No significant toxicity of the Desferal-ascorbic acid regimen is clinically obvious. Analysis of all the data collected over the past four years is planned during the next several months.

Significance to Biomedical Research and to Institute Program:

Hemosiderosis is a major cause of morbidity and mortality in patients requiring prolonged transfusion therapy. The role of iron chelators in improving the clinical course of these patients must be ascertained.

Proposed Course of the Project:

This project will be continued until a suitable iron chelator is found and evaluated or until the need for transfusion therapy in thalassemia and other congenital hemolytic anemias is removed. The majority of our series of thalassemic patients on the chelation trial have had three or more annual evaluations. Within the next several months we anticipate analyzing the followup studies and comparing the results to baseline data with particular attention to cardiac function. From this analysis we hope to learn whether there is any difference in the clinical course of the patients on ascorbic acid compared to those on placebo and therefore to determine whether this phase of the trial need be continued.

Publications:

1. Nienhuis, A.W. and Griffith, P.: Thalassemia. In Gellis, S.S. and Kagan, B.M. (Eds.): Current Pediatric Therapy. Tenth Edition, W.H. Saunders, Phila. 1981.

2. Nienhuis, A.W.: The Thalassemias. In Wyngaarden, J.B. and Smith, L.H., Jr. (Eds): Cecil Textbook of Medicine, Sixteenth Edition, W.B. Saunders, Phila. 1982. pp. 882-885.

3. Cannon, R.O., III, Dusheiko, G.M., Long, J.A., Ishak, K.G., Kapur, S., Anderson, K.D. and Nienhuis, A.W. Hepatocellular adenoma in a young woman with β-thalassemia and secondary iron overload. Gastroenterology 81: 352, 1981.

4. Ley, T.J., Griffith, P., Nienhuis, A.W. Transfusion Hemosiderosis and Chelation Therapy. In Jacobs, A. and Wolvey, W. (Eds.) Clinics in Haematology, Volume II, No. 2, 11:437-464, 1982. W.B. Saunders Co., Ltd., Eastbourne, East Sussex.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02304 05 CHB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Hematopoiesis in Bone Marrow Failure

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Pedro Gasco	Staff Fellow	CHB	NHLBI
Warren Ferris		CHB	NHLBI
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Philip Mortimer			
Jeffrey Moore		CHB	NHLBI
Harvey Klein		BB Dept	NHLBI

COOPERATING UNITS (if any)
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Blood Bank, Clinical Center, NIH

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	3	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Insights into the mechanism of normal and aberrant hematopoiesis have been provided by the study of patients with bone marrow failure and their tissues. Patients with aplastic anemia have been treated in the Clinical Center with drug immunosuppression consisting of lymphocytophereis, plasmapheresis, cyclophosphamide, and prednisone; the best hematologic responses have been achieved with antithymocyte globulin. Studies of the tissues of patients with aplastic anemia have disclosed an abnormal ratio of helper to suppressor lymphocytes in the peripheral blood, but this ratio remains abnormal even with hematopoietic recovery. It has proven extremely difficult to demonstrate a suppressor lymphocyte using monoclonal antibodies in tissue culture systems. Helper lymphocytes are decreased significantly in aplastic anemia. In addition, natural killer cell function is also deficient, possibly as a result of multiple transfusions. The role of cell-cell interaction in bone marrow failure thus remains uncertain. However, strong, evidence has been developed that in transient aplastic crises that occur in children with sickle cell anemia, the mechanism of suppression is direct infection of bone marrow with a human parvovirus-like agent.

Objectives:

The continuous production throughout life of blood platelets, granulocytes, and erythrocytes by bone marrow stem cells is dependent upon appropriate interactions of cells and humoral factors in the bone marrow.. Using in vitro tissue culture systems, evidence has been developed that certain helper lymphocytes are required for hematopoiesis, possibly in combination with monocytes. Less certain is the normal role of suppressor cells, either lymphocytes or natural killer cells, in bone marrow failure and in the normal regulation of populations with high self renewal capacity. By analogy with other diseases mediated by immunological mechanisms, certain forms of bone marrow failure may be immunologically mediated. The immunological suppression which precedes bone marrow transplantation has resulted in recovery of autologous bone marrow function in some patients. An occasional syngeneic twin has been unable to accept his bone marrow transplant in the absence of such immunosuppressive therapy. Studies have reported the high incidence of either suppressor lymphocytes in patients with aplastic anemia or humoral factors that inhibit hematopoiesis in vitro. Most impressive, European and, recently, American trials of anti-thymocyte globulin have shown that approximately 50% of patients with severe aplastic anemia will demonstrate a hemotologic response to this serum preparation; these response rates have challenged those obtained by bone marrow transplantation.

We have accumulated substantial experience in the treatment of patients with severe aplastic anemia. Initially, patients were treated with apheresis (plasmapheresis or lymphocytopheresis) in combination with cyclophosphamide and prednisone. An attempt was made to correlate the rate of responsiveness in patients with aplastic anemia and pure red cell aplasia to this regimen and changes in cellular and humoral indicators of suppression in vitro. During the past year, a larger number of patients have been treated with anti-thymocyte globulin with or without high doses of the corticosteroid, methylprednisolone. During this study, attention has been directed towards the quantitation of helper and suppressor lymphocyte cell populations and natural killer cell function, and their modulation by ATG therapy. Finally, using virus obtained from human subjects infected with a parvovirus-like agent, compelling evidence has been developed that some cases of bone marrow failure may be directly due to infection of hematopoietic progenitor cells. The implications of viral effects on hematopoiesis, both through cytotoxicity and by stimulation into differentiation pathways, have been explored using tissue culture techniques.

Methods:

Treated patients with aplastic anemia have fulfilled the criteria for severe disease, as defined by peripheral blood counts in the presence of a hypoplastic bone marrow biopsy. Patients with pure red cell aplasia and amegakaryocytic thrombocytopenia have also undergone treatment, and the family members of patients with aplastic anemia as well as individuals with moderate or recovered bone marrow aplasia have been studied. Patients treated by apheresis and drug immunosuppression underwent plasmapheresis and lymphocyte depletion three times weekly for one month in combination with cyclophosphamide (2 mg/kg/day) and prednisone (1 mg/kg/day). Patients treated with antithymocyte globulin received the Upjohn preparation, derived from the sera of horses immunized with human thymocytes. Treatment consists of 15 mg/kg/day Upjohn ATG for 14 days and then the same dose administered on alternate days for a further 14 days. Some individuals have also been treated with methylprednisolone at doses beginning at 20 mg/kg/day intravenously and gradually tapered over one month. The androgen, oxymethylolone, has been given at doses of 2 mg/kg/day during the treatment. Immunosuppression

and hematologic effects have been measured by serial blood counts, serum chemistries, quantitative immunoglobulins, complement, and responsiveness to skin tests.

Hematopoietic colonies have been grown in methylcellulose in the presence of placental condition medium to stimulate myeloid colony development or phytohemoglutinin stimulated leucocyte conditioned medium and erythropoietin to stimulate erythroid colony growth. Our conditions are such that removal of the adherent cells, which normally produce either colony stimulating or burst promoting activity, affects neither the size nor number of colonies formed. Serum inhibitors of hematopoiesis have been assayed using bone marrow or peripheral blood cells from normal donors and sera from patients, in the presence of autologous human serum as a source of complement. For studies of the effects of viral preparations on hematopoiesis, virus-containing sera or cultures have been incubated for 4 hours at 4°C with normal human bone marrow cells, which are then assayed by colony formation. To assess the effects of depletion of specific lymphocyte subpopulations, commercially available monoclonal antibodies against helper lymphocytes, suppressor lymphocytes, natural killer cells, and monocytes have been incubated with normal or aplastic bone marrow in the presence of complement prior to plating. Colonies derived from the myeloid precursor, CFU-C, are counted at 10 days. Colonies derived from the CFU-E, the late hematopoietic preogenitor, consisting of at least 8 hemoglobin-containing cells in a tight cluster, are determined at 8 days, and colonies derived from the most primitive erythroid progenitor, the BFU-E, are counted at 12 and 18 days. Careful attention is also paid to the qualitative appearance of hematopoietic colonies, especially in the presence of virus. Bone marrow cells have also been grown in liquid culture; under normal circumstances, proliferation of hematopoietic cells occurs during the first week of culture resulting in an increase in the number of colony forming cells. This increase is a reflection of either movement of stem cells into the committed progenitor compartment or self-renewal within the colony forming unit population. The cycling rates of progenitor cells have been measured using standard tritiated thymidine suicide techniques. Finally, a measure of the grown factor producing potential of peripheral blood leukocytes has been measured by comparison to normal controls. In these experiments, peripheral blood leukocytes of normal persons and patients are incubated for one week in liquid culture, the supernatant is harvested and subsequently tested for its ability to stimulate colony formation by non-adherent cells of bone marrow.

Immunological studies have consisted of the measurement of the number of helper, suppressor, natural killer, B cell, monocytes, and T cell in the peripheral blood of normals and patients with aplastic anemia. The fluorescent activated cell sorter (Becton-Dickinson FACS II) has been used for the analysis of peripheral blood. Ficoll-Hypaque purified lymphocytes are incubated with appropriate commercial monoclonal antibodies directed against cellular subtypes and fluorescent anti-mouse immunoglobulin. The FACS allows for reproducible and rapid quantitation of the number and relative proportion of these cells in the peripheral blood. A technique employing immunoperoxidase staining of frozen bone marrow biopsy sections has been developed to measure helper and suppressor lymphocytes in bone marrow. Natural killer cell function has been measured using Cr-labeled K562 cells, direct binding of fluoresceinated lymphocytes to K562 cell target, and FACS analysis using a natural killer cell-specific monoclonal antibody.

Two general types of virus preparations have been used in hematopoietic colony culture studies. First a parvovirus-like agent present in the sera of otherwise normal blood bank donors and also detected during the acute phase of non-specific viral infections has been used for studies of inhibition of hematopoiesis. This parvovirus has been implicated by immunological assays in aplastic crisis of sickle cell disease. In other studies, human bone marrow cells have been incubated with Harvey and Kirsten virus preparations. These viruses have been shown to stimulate murine cells to differentiate in the absence of conventional growth factors like erythropoietin.

Results:

A. Clinical trials of immunosuppression and ATG-methylprenisolone.

As reported in the Annual Report of 1981, plasmapheresis and lymphocyte depletion in combination with cyclophosphamide and prednisone resulted in clinical responses in three patients with pure red cell aplasia but in only one of six patients with aplastic anemia. In contrast, 14 patients have now been treated with ATG with or without high doses of methylprednisolone. Two patients showed complete hematological recovery; one of these patients had had stable bone marrow depression for over one year prior to treatment. Two other patients demonstrated significant but transient increases in blood counts. Four patients died before completing a full course of ATG therapy. If only the patients who were evaluated at the completion are included, the response rate is similar to that reported in the literature. An important finding has been that methylprednisolone in the doses advocated by other workers for the treatment of acute, severe aplastic anemia, has significant toxicity: marked susceptibility to fungal and viral infections, metabolic abnormalities such as insulin dependent diabetes, and aseptic necrosis of the femoral head.

B. Lymphocytes and natural killer cells in aplastic anemia.

In normal individuals, the ratio of helper to suppressor in the peripheral blood lymphocytes; as defined by monoclonal antibodies, is approximately 1.5. However, in patients with severe aplastic anemia, this ratio is reversed (mean 0.4). The degree of abnormality of the helper/suppressor lymphocyte ratio approximately correlates with the severity of bone marrow depression. This abnormal ratio is the result of a decrease in the number of helper cells, while the number of suppressor lymphocytes is not statistically different from normal. Suppressor lymphocytes have been implicated in functional studies of the effects of aplastic anemia patients' bone marrow and peripheral blood on normal hematopoiesis. With response to ATG therapy, the abnormal helper suppressor ratio does not change. Attempts to deplete aplastic anemia patients' bone marrow using monoclonal antibodies and demonstrate either enhanced colony formation in the absence of suppressor cells or decreased colony formation in the absence of helper cells have been unsuccessful. In the bone marrow, suppressor lymphocytes outnumber helper lymphocytes in normal individuals, and the relative ratio is only slightly increased in favor of suppressor cells in patients with aplastic anemia. These studies have cast doubt on the hypothesis that aplastic anemia is the result of an abnormal clone of suppressor cells in hibiting hematopoiesis.

The natural killer cell is another cell capable of suppression of hematopoiesis by cytotoxic activity. Surprisingly, natural killer cells were found to be virtually absent in patients with aplastic anemia. In heavily transfused individuals with thalassemia and sickle cell disease, natural killer cell activity was also very low. However, the number of natural killer cells as

measured by NI-1 specific monoclonal antibody, was not found to be abnormal in patients with aplastic anemia. This functional deficiency of natural killer cells is at the moment inexplicable, but does not appear to be the result of either splenectomy or hematologic disease as such.

C. Paroxysmal nocturnal hemoglobinuria.

Patients with PNH have a peculiar form of bone marrow failure. This disease has been thought to be the result of an abnormal sensitivity of peripheral blood erythrocytes to the effects of complement. Patients have a variable number of such hypersensitive cells in their circulation. A larger proportion of patients with PNH succumb to true bone marrow aplasia. These patients have shown a remarkable decrease in the number of myeloid and erythroid hematopoietic progenitors in peripheral blood. Both bone marrow and blood colony numbers are in the range observed in severe aplastic anemia. The in vitro results do not seem to be the result of abnormal hypersensitivity to complement in vitro. These results suggest that patients with PNH, analogous to individuals with congenital aplastic anemia, have evidence of severe bone marrow dysfunction long before marrow aplasia develops; it is likely that the near normal hematopoiesis observed clinically is supported by a very small number of normal hematopoietic stem cells.

D. Virus effects on hematopoiesis.

A serum parvovirus-like agent (SPLA) has been described in the blood of normal blood donors and patients with non-specific viral illnesses. Antibody to the virus has been detected in 60 to 70% of normal individuals and in the convalescent sera of children recovering from transient aplastic crises. We have shown that sera containing SPLA is cytotoxic to erythroid progenitors, as measured in the tissue culture. The potency of this virus is high; using serum obtained from a patient who developed aplasia in the setting of hereditary spherocytosis, cytotoxicity was demonstrable at a final concentration of 1:1000. The effect of the virus appears to be predominantly on cell at the CFU-E stage; CFU-C are unaffected. Inhibition is reversible by convalescent serum containing antibody. Preliminary evidence has suggested that susceptible cells may be characterized by a high cycling capacity. In addition, a helper virus which is heat sensitive is likely also involved in the infectious process. Serum parvovirus-like antigen has not been detected in serum obtained from patients with adult aplastic anemia, nor is the incidence of antibody higher in these patients than in the normal control population. However, a virus similar to the parvovirus or another virus may have a similar mechanism of action in patients with aplastic anemia.

Genes present in the human genome, homologous to viral DNA sequences in animals associated with carcinogenesis, have been implicated as important in the normal proliferation or differentiation of hematopoietic cells. The Harvey and Kirsten viruses have been shown, in mouse tissue culture systems, to result in erythroid differentiatiion in the absence of growth factors. Similar studies have been performed using these virus preparations in human bone marrow cells, but no effects on differentiation have been demonstrated. This difference between human and murine systems may be the result of the absence of a specific human promoter sequence in these viruses.

Proposed Course of the Project:

The Upjohn Company has agreed to fund a multicenter trial of its antithymocyte globulin, which will be directed by the Clinical Hematology Branch of the National Heart, Lung, and Blood Institute. Twelve institutions in the United States will cooperate in treating patients with aplastic anemia and other hemato-

logic diseases. Two drug regimens will be compared in patients with severe aplastic anemia. ATG treatment will be contrasted to therapy with androgens and transfusions in patients with chronic or moderate aplastic anemia. Serum and peripheral blood cells will be made available to the Hematology Branch for viral and immunological studies. There will be a large scale examination of the features of the serum sickness that develops uniformly in ATG-treated individuals.

The role of natural killer cells in normal and aberrant hematopoiesis is being further investigated. A large number of patients who have undergone transfusions are being assayed for natural killer cell function. Functional activity is being compared to NK number as determined by cell sorting. Changes in natural killer cell function which result from transfusion are being examined in patients with hematologic disease and individuals undergoing cardiac surgery. In the laboratory, the effects of natural killer cells on autologous bone marrow function are being examined using tissue culture techniques to search for a normal surveillance role for stem cells. The role of growth factors such as interleukin 1, interleukin 2, and interferon in patients with aplastic anemia will be examined using specimens obtained from the cooperative trial.

The human parvovirus like agent which infects hematopoietic cells has not been cultured in vitro. Attempts are underway to establish long term cultures using normal hematopoietic cells from humans. The mechanism by which the virus infects erythroid cells may be the result of binding to glycophorin on the erythrocyte membrane. More sensitive screening methods for antibody to parvovirus will be employed in patients with aplastic anemia. A search for related viruses or evidence of viral infection, such as elevated interferon levels, will be examined in the large number of patients admitted to the multicenter trial.

Publications:

1. Young, N.S.: Aplastic Anemia: Research Themes and Clinical Issues. In Progress in Hematology, Vol XII (ed.) elmer B. Brown, Publisher - Grune & Stratton, Inc. 1981.

2. Young, N.S., Klein, H.G., Griffith, P., and Nienhuis, A.W.: A Trial of Immunotherapy in Aplastic Anemia and Pure Red Cell Aplasia. J. Clin. Apheresis, Submitted.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL02307 03 CHB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Use of Viral Regulatory Sequences to Facilitate Gene Transfer and Analysis of Gene Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI -	Keith Humphries	Visiting Associate	CHB NHLBI
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COOPERATING UNITS (if any)
None

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS X ☐ (b) HUMAN TISSUES X ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

These studies have focused on the immediate goal of defining regulatory sequences involved in the normal expression of globin genes. Plasmid vectors containing the human α, β or δ globin genes were constructed to study globin gene expression following introduction of these recombinant vectors into cultured mammalian cells (monkey kidney Cos cell line) by the calcium phosphate precipitate technique. Function of the δ and β but not α globin gene promoter required enhancer sequences provided by the tandemly repeated 72 bp sequences of the SV40 viral genome. Further differences in promoter function were revealed by the 50-fold higher level of expression of the β globin gene compared to the δ globin gene. Application of the expression system to the study of thalassemic β globin genes confirmed the utility of this system for quantitation of gene expression and for analyzing RNA processing defects. Insights into globin gene regulation gained with this approach should provide a rational basis for construction of vectors designed to facilitate DNA transfer and regulated gene transcription in mammalian cells.

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PHS-6040
(Rev. 2-81)

The advent of the techniques of molecular cloning and recombinant DNA technology have resulted in remarkable advances in our knowledge about gene structure and function. With such knowledge the goal of correcting human genetic defects which result in severe disease by genetic therapy becomes increasingly more realistic. To reach this goal however, it must be recognized that while much is known about DNA sequence and gene organization on chromosomes, the sequences and mechanisms involved in regulating gene expression have not been fully defined. Ultimately, functional studies are required to test the significance of presumed regulatory sequences and to provide insight into other possible control regions and mechanisms. Recently such studies have been facilitated by the development of short term expression systems which are based on the introduction of recombinant plasmid vectors into mammalian cells. This strategy combines the ease and flexibility of recombinant plasmid constructions in bacteria with the potential for rapid high level expression in eukaryotic cells, previously associated only with the use of recombinant viruses.

We have constructed plasmid vectors and utilized such an expression system to study human globin gene regulation. A series of vectors were constructed which replicate both in E. coli and mammalian cells. All vectors contain a portion of SV40 genome required for DNA replication. When such vectors are introduced into a line of monkey kidney cells which constitutively produce SV40 T antigen, amplification of the vector sequences occurs. The vector is presumably packaged into nucleosomes forming a mini-chromosome which is equivalent to the natural template for RNA synthesis. Also studied were vectors containing additional sequences of SV40 encompassing the 72 bp directly repeated sequences which appear to have an enhancing effect on the function of some promoters. Expression of the human α, β or δ globin genes were compared using vectors with or without the SV40 enhancing element. In addition, hybrid $\beta\delta$ and $\delta\beta$ globin genes were analyzed to determine which sequences are responsible for hypo function of the δ globin gene. Finally, the system was used to help delineate the nature of the defect in abnormal globin genes isolated from two patients with β^+ thalassemia.

Methods:

1. Construction of expression vectors: All constructions are performed by use of the plasmid vector, pBR322, to allow cloning of constructs into E. coli. Using recombinant DNA techniques, an expression vector was constructed by removing from pBR322, sequences which inhibit its repliction in mammalian cells and by adding the SV40 origin of repliction. Cloned DNA fragments containing the human δ, β, or $\alpha 2$ globin genes were then inserted into this expression vector. These recombinants were then modified by addition of a region of SV40 containing the 72 bp direct repeat elements flanked bilaterally by SV40 fragments containing the early mRNA poly-adenylation sites. The later sequences served to isolate functional SV40 early and late promoters from the globin gene promoter also present in the vector.

2. Introduction of plasmid expression vectors into monkey kidney cells: Purified plasmid DNA was mixed with a carrier salmon sperm DNA and precipitated by incubation in a solution containing calcium phosphate. This precipitate was added to the monkey kidney COS cell line (which constitutively produces T antigen) and after four hours cells were treated with a glycerol solution to increase transformation frequency. After removal of the DNA precipitate , the cells were further incubated for 48 hours and DNA and RNA were recovered.

3. Analysis of plasmid vector replication: Plasmid replication in COS cells was assessed by extracting low molecular weight DNA from replicate dishes

by the protocol of Hirt, 48 hours aftr transfection. Following purification, the DNA was electrophoresed on agarose gels directly or after restriction digestion with Dpn I. Following transfer of DNA to nitrocellulose filters by the Southern blot technique, plasmid sequences were visualized by hybridization to globin cDNA probes. Resistance of plasmid DNA, recovered 48 hours after infection, to digestion by Dpn I verified that plasmids contained a eukaryotic methylation pattern and thus represented molecules newly replicated in COS cells. In experiments where globin gene expression in different vectors was compared, spot blot analysis of DNA extracted from separately infected populations of cells was performed to determine relative plasmid copy number.

4. Analysis of globin gene RNA transcripts: The amount and nature of globin transcripts generated from different vectors was extensively analyzed using three techniques. First, total COS cell RNA, recovered 48 hours after transfection, was annealed in solution to probes specific for globin mRNA sequences to allow their quantitation. Second, the size of globin RNA species was determined by gel electrophoresis and Northern blot analysis. Third, globin gene transcripts were extensively mapped with respect to initiation site, processing of intervening sequences, and termination site. Using a new technique developed in this lab, probes were synthesized from templates produced by recombination of the single stranded filamentous bacteriophage M13mp7 with fragments of the human δ, β or α globin genes. Mapping was done by annealing RNA to uniformly labeled single stranded probes corresponding to defined regions of a globin gene. Following S_1 nuclease digestion, protected probe fragments were resolved by gel electrophoresis.

5. Construction of $\beta\delta$ and $\delta\beta$ globin hybrid genes: To better define the region of the δ globin gene fragment responsible for the low levels of mRNA found in COS cells, various hybrids of the human δ and β globin gene were created. Two hybrids were constructed in which different amounts of the δ globin gene 3' region were replaced with a comparable portion of the β globin gene. A $\beta\delta$ hybrid gene was made in which the 3' region of the δ gene was replaced by a corresponding fragment from the β gene.

6. Determination of DNAse I hypersensitive sites in expression vectors: 48 hours after transfection of monkey kidney cells, nuclei were isolated and exposed to variable concentrations of DNAse I. The DNA was then purified and digested with a restriction enzyme. Gel electrophoresis and Southern blot analysis were then performed to detect specific sites in plasmid expression vectors selectively cut by DNAse I.

7. Application of expression system to study defective β globin genes: Globin genes cloned from two β^+ thalassemic patients were inserted into the plasmid expression vector containing the SV40 enhancer element. A number of hybrid genes formed by fusion of thalassemic and normal β globin genes were constructed and inserted into the expression vector. Following introduction of these vectors into monkey kidney cells, β mRNA was quantitated and analyzed as described above.

Results:

1. Replication of plasmid expression vectors in COS cells. Replication of the various plasmid vectors in COS cells was demonstrated by resistance of plasmid DNA, recovered 48 hours after infection, to digestion by Dpn I. By 48 hours each infected COS cell contained 3000 to 10,000 copies of plasmid vector thus providing many templates for RNA transcription.

2. Comparison of the δ, β and α globin gene promoters. When the human α globin gene was introduced into COS cells using the basic vector lacking the 72 bp repeats of SV40, high levels of α mRNA were synthesized. This α mRNA was found to be normally initiated, processed, and polyadenylated and was indistinguishable from normal α mRNA present in bone marrow cells. Addition of the segment of SV40 containing the 72 bp direct repeat enhancer element had no effect on α globin gene expression. In marked contrast, virtually no correctly initiated transcripts of the δ or β globin gene were generated by vectors lacking the SV40 enhancer element. In such vectors, the δ and β globin gene promoters are inactive. However, addition of the SV40 72 bp repeat element resulted in normally initiated transcription from the δ and β globin genes indicating activation of the δ and β globin gene promoters. Vectors in which the 72 bp repeats were specifically deleted resulted in loss of β globin gene expression, directly demonstrating that this region is responsible for the enhancing effect on promoter function.

In vectors containing the 72 bp repeats and the δ or β globin gene, the activity of the β globin gene was approximately 50 times greater than the δ globin gene, approximating the ratio of δ and β mRNA observed in normal human bone marrow cells. Analysis of hybrid βδ or δβ genes indicated that decreased expression of the δ globin gene is attributable to hypofunction of the δ promoter region.

3. Analysis of promoter activation. The correct function of the α gene promoter in vectors lacking the SV40 enhancer element suggests that the α globin gene may contain sequences which perform an enhancer function. This hypothesis was directly tested by studying vectors containing both the α and β globin genes. The α promoter was active but the β globin promoter did not function in such vectors. These studies have not comletely ruled out the presence of an enhancer element in the α globin gene segment however. Indeed the presence of enhancer elements in eukaryotic genomes and their potential relevance to in vivo globin gene expression remains unresolved.

Analysis of DNAse I hypersensitive sites in the globin gene expression vectors revealed one site in the region of the SV40 origin of replication near the 72 bp repeats - a site similarly seen in intact SV40 virus. To date no significnt differences in the number and location of hypersensitive sites flanking the α or β globin gene in these vectors, or activation of hypersensitive sites with the addition of the SV40 enhancer elements have been found. These studies thus leave unexplained the difference in α and β globin gene promoter function and the mechanism of promoter activation by enhancer sequences.

4. Application of the expression system to study of defective thalassemic globin genes. β globin genes obtained from two patients with β^+ thalassemia have now been studied with this expression system. The expression of these genes relative to a normal β globin gene was reduced, reproducing the in vivo situation. Study of a variety of recombinants between thalassemic and normal globin genes in conjunction with DNA sequencing have allowed the definitive identification of those sequences responsible for the defective function. (See Individual Project: Molecular Defects in thalassemia).

Significance to Biomedical Research and the Institute Program.

Many serious human diseases arise because of monogenetic mutations affecting the structural or enzymatic component within cells. Two of these in which we are particularly interested are sickle cell anemia and homozygous β thalassemia. The goal of achieving genetic therapy for these conditions should be attainable with increased knowledge of globin gene regulation. In the course of pursuing this objective, additional knowledge will be gained regarding the function of transcriptional regulatory signals in eukaryotic cells.

Proposed Course of the Project

Several immediate goals can be defined.

1. We will continue to define the genetic elements essential for correct globin gene transcription and thus provide a rational basis for construction of vectors potentially useful for gene transfer. A search will be made for sequences which normally enhance globin gene promoter function in vivo. Repetitive sequences present near the β globin gene complex will be screened for enhancing activity and further recombinant molecules containing the α and β globin genes will be analyzed to look for enhancing sequences in the α globin gene.

2. Sequences upstream to the δ globin gene will be tested in these vectors for inhibitory effect on gene expression. The existence of such "down regulators" has been suggested by our results and from study of patients with deletions in the region of the non-α globin genes.

3. We will continue to use the system to study thalassemic globin genes in particular those genes tentatively identified from other studies, as possibly representing novel mutations such as defects in the promoter region.

4. Expression vectors constructed so as to contain known essential elements for globin gene transcription will be introduced into intact mice by the technique of microinjection of DNA in the pronucleus of fertilized mouse eggs. Integration of such vectors and subsequent development of a "transgenic" mouse will allow the study of gene expression during normal development. Different tissues will be examined to determine whether tissue specific and normal levels of expression occurs during ontogeny and erythroid cell differentiation.

Publications:

1. Nienhuis, A.W., Ley, T.J. and Humphreies, R.K.: Expression of normal human and thalassemic globin genes. Proceedings of "The Regulation of Hemoglobin Biosynthesis:" edited by Goldwasser, E.

2. Humphries, R.K., Ley, T., Turner, P., Moulton, A.D. and Nienhuis, A.W.: Differences in human alpha, beta and delta globin gene expression in monkey kidney cells. Cell, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02308 03 CHB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Characterization of Repeated DNA Sequence Family, a Member of Which is Found Near the Human β Globin Gene

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Jesse Adams	Clinical Associate	CHB	NHLBI
Other:	Amanda Cline	Chemist	CHB	NHLBI
	Arthur W. Nienhuis	Branch Chief	CHB	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.25	0.25

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A family of long interspersed repetive DNA sequences in the human genome has been characterized utilizing a member of the family present immediately 3' to the human β globin gene. 3000-4800 other members of this family are dispersed throughout the genome. In situ hybridization, using a portion of the member of the repeat family adjacent to the β globin gene as a probe, has revealed that the family is represented more or less uniformly on all human chromosomes with no evidence of centromeric localization. The sequence homology of 3 members of the family has been defined precisely by DNA sequencing of the 5' end; one member precisely abuts another characterized repetitive DNA element, whereas other members of the 6.4 kb family are found in DNA which is not highly repetitive. A small portion of the member of the repeat found near the β globin gene appears to be weakly homologous to sequences found near the SV40 origin of replication. Experiments are in progress to determine whether there are enhancer-like elements, analogous to the 72 bp direct repeats in the SV40 genome, within individual members of the 6.4 kb repeat sequence family.

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Objectives

Repeated DNA sequences have been postulated to be related to transposable elements which are found in prokaryotes and in lower eukaryotes. Transposable elements of these species may mediate DNA mutations including deletions and rearrangements, and may play a similar role in human DNA thereby precipitating both genetic and neoplastic diseases. We are characterizing a family of long interspersed repeated DNA sequences, found in the human genome. Ultimately, we hope to learn whether such sequences have an important functional role in gene regulation or whether they are an evolutionary by product of the capacity of eukaryotic cells to rearrange their DNA.

Methods:

1. In situ hybridization: A DNA fragment derived from the member of the repeat sequence family found adjacent to the human β globin gene served as a template for synthesis of a [^{3}H] copy RNA. This cRNA was used as a probe against human metaphase chromosome spreads to determine the distribution of the individual members of the repetitive DNA sequence family with respect to the human chromosomes.

2. DNA Sequencing: DNA fragments from different members of the 6.4 kb repeated sequence family were subcloned into the plasmid vector, pBR322, and DNA sequencing was performed using both the Maxam-Gilbert and M13-dideoxy DNA sequencing techniques.

3. Search for enhancer sequences: DNA fragments containing a portion of the 6.4 kb repeated sequence adjacent to the β globin gene were subcloned into a plasmid expression vector containing a truncated version of the human β globin gene. The various recombinant plasmids were separately introduced into COS cells (see individual project "Use of Viral Regulatory Sequences to Facilitate Gene Transfer and Analysis of Gene Function"). COS cells constitutively produce SV40 antigen allowing replication of the expression vector and expression of the genes contained therein. The RNA transcription products are assayed with respect to their 5' end; the fraction of β globin RNA molecules which are initiated at the normal β globin promoter provides a measure of enhancer function.

Major Findings:

1. The individual members of the six long interspersed repetitive DNA sequence family we have characterized appear to be uniformly distributed over the human chromosomes. Lack of centromeric localization confirms that these sequences are unrelated to satelite DNA. The interspersion pattern is consistent with a functional role for these sequences in modulating gene expression.

2. Sequencing of the 5' end of three copies of the 6.4 kb repeat family, when combined with Southern blotting experiments, verify that the relationship between the Alu and the 6.4 repeat near the β globin gene is apparently fortuitous. No specific structural relationship between individual members of the two repeat sequence families is evident.

3. A portion of the member of the repeat sequence family found 3' to the human β globin gene shows weak hybridization to a DNA fragment containing the SV40 origin of replication. The nature of this sequence homology and the in-

fluence of this DNA fragment on function of the human β globin gene promoter is being studied in an in vivo expression system.

Significance to Biomedical Research and the Institute Program:

Much DNA in the human genome does not encode for a protein or an RNA product. Such DNA has no known function. Much of the non-coding DNA in the eukaryotic cell genome appears to be repetitive. We are focusing on a well characterized repeat sequence family in the human genome to learn whether individual members of the family influence the function of genes in the immediately surrounding DNA. The human β globin gene provides a model for this study since there is a member of this repeat sequence family very close to this gene.

Proposed Course of the Project:

The recombinants, containing portions of the repeat sequence family and the human β globin gene will be studied in the in vivo expression system within the next several months. If enhancer effects are defined in specific fragments, our intent is to precisely localize the enhancer region by construction of a series of recombinants containing smaller portions of the repeat family. Once the activity is localized to a relatively small fragment, the DNA sequence of this fragment will be determined and its homology to other enhancers will be defined.

Publications:

1. Adams, J.W., Kaufman, R.E., Kretschmer, P.J., Coon, H.C., Anderson, W.F., and Nienhuis, A.W.. Cloning and Characterization of DNA Sequences Surrounding Human β-like Globin Genes: Identification of an Unusually Long Moderately Repetetive DNA Sequence. In "Organization and Expression of Globin Genes". Stamatoyannopoulos, G. and Nienhuis, A.W., Editors. Alan R. Liss, Inc. 1981 pp 33-44.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02309 03 CHB

PERIOD COVERED

October 1, 1981 - September 30, 1982

TITLE OF PROJECT (80 characters or less)

Use of Hybridoma Technology in the Study of Erythroid Differentiation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Neal S. Young	Senior Investigator	CHB NHLBI
Other:	Pedro Gascon	Staff Fellow	CHB NHLBI
	Robert Gallagher	Senior Investigator University of Maryland Cancer Center Baltimore, Maryland	
	Keith Humphries	Visiting Associate	CHB NHLBI

COOPERATING UNITS (if any)

University of Maryland Cancer Center, Baltimore, Maryland

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1-1/2	1-1/2	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Monoclonal antibodies produced by hybridoma lymphocytes can have high specificity for cell membrane antigens present on distinctive cell types. Hybridoma technology has been employed in our laboratory to produce antibodies which bind selectively to hematopoietic progenitor cells. Because these cells are unavailable in quantities sufficient for immunization, the K562 leukemia cell has been employed as an antigen because it has properties similar to those of the true hematopoietic stem cell. Antibodies raised in mice against K562 cells show a variety of binding patterns to normal cells, as assayed by various immunological methods. A small number of these antibodies are inhibitory to hematopoietic colony formation. At least one antibody has the capacity to bind to the myeloid progenitor, as demonstrated by fluorescent activated cell sorting. Two anti-K562 monoclonal antibodies have been extensively characterized by immunochemical and biological means. These antibodies recognize antigens characteristic of the undifferentiated state of myeloid cells.

PHS-6040
(Rev. 2-81)

Objectives:

Antibodies produced by hybridoma technology have two important advantages over conventional antisera. First, large quantities of monoclonal antibodies can be produced because the hybridoma cell lines are "immortal". Second, the procedure for choosing the interesting antibody-producing clones allows for selection of unusual or rarely represented specificities that would be obscured in analysis of an antiserum. For example, the hematopoietic colony assay allows for the selection of antibodies which bind to hematopoietic progenitors. Thus this method appears particularly suitable for the production of antibodies to the rarely represented hematopoietic stem cell and the related hematopoietic progenitor cells that are responsible for colony formation in vitro. The K562 cell has been used as an immunogen because it may share antigenic determinants with stem cells. The K562 cell expresses not only granulocytic but also erythroid, megakaryocytic, and lymphocytic cell phenotypic characteristics. The K562 cell may be induced to produce embryonic and fetal hemoglobins and to form small erythroid-like colonies in semi-solid media. The combined properties of "pluripotency" and the capacity for indefinite self-renewal are analogous to those of the hematopoietic stem cell. The K562 cell was chosen for immunization of mice in an effort to produce antibodies against hematopoietic stem cells present in human bone marrow.

The use of such monoclonal antibodies for the separation of infrequently represented hematopoietic progenitors in the bone marrow has proven difficult for several reasons. Biologically, it appears that very few if any monoclonal antibodies have absolute specificity for immature cells. Second, the slow rate of sorting in the fluorescent activated cell sorter and the difficulty of using larger scale methods for isolation of a small percentage of cells are important technical obstacles. Monoclonal antibodies to hematopoietic cells may prove useful for other purposes besides cell separation. They may serve as markers of the undifferentiated state, and improve classification of diseases and in the detection of hematopoietic progenitors by immunological methods in bone marrow specimens. Second, the function of cell surface proteins that are most heavily represented in primitive cells may be elucidated using these antibodies. Third, the antibodies have been useful in elucidating the mechanism by which natural killer cells attack and destroy K562 cell targets.

Methods:

Anti-K562 cell monoclonal antibodies were originally prepared from the fusion of spleen cells of Balb/C mice and SP/2 myeloma cells. Eight monoclonal antibody lines have proven to be long-term antibody producers, and these cells have been employed in the production of large amounts of hybridoma supernatant and ascites fluid. The binding of anti-K562 cells and monoclonal antibodies to normal and malignant human cell types have been assayed using a variety of immunological techniques, including complement mediated cytotoxicity, microscopic immunofluorescence, fluorescent activated cell sorting, and radioimmunoassay. The effects of monoclonal antibodies on human bone marrow and tissue culture and K562 cell colony formation in methylcellulose have also been examined. Molecular weights of antigens recognized by two monoclonal antibodies have been determined by immunoprecipitation with staphylococcal A.

Results:

Two monoclonal antibodies directed against K562 cells have been extensively characterized. These antibodies were selected for further study because of their binding properties to HL-60 cells. This leukemic cell line has the property of differentiating to a granulocytic phenotype in the presence of inducers like dimethylsulfoxide and retinoic acid. This cell line was useful in testing monoclonal antibodies for their ability to bind preferentially to immature in contrast to differentiated myeloid cells. Antibody 76/59 had previously been shown to inhibit the formation of myeloid colonies derived from the CFU-C. 80/97 was a second antibody studied. Both antibodies demonstrated high binding to HL-60 cells prior to induction of differentiation. The fluorescent activated cell sorter was employed to analyze the population of cells to which the antibodies bound; it was found that a decrease in radioimmunoassay binding correlated with the percentage of cells which differentiated to polymorphonuclear-like cells. Antibody 80/97 had the peculiar property of being the only monoclonal antibody tested that showed binding to a retinoic acid resistant HL-60 cell line. In the presence of DMSO, but not in the presence of retinoic acid, 80/97 binding decreased on induction of differentiation. Therefore, it was concluded that both antibodies recognized antigens that were present on early or immature myeloid cells and that these antigens decreased or changed character as cells differentiated.

The binding properties of these two antibodies to other cell lines were examined using both the fluorescent activated cell sorter and radioimmunoassay. 76/59 has specificity for hematopoietic cells, binding to cell lines derived from myeloid leukemia, monocytic tumors, pre-B and pre-T as well as undifferentiated B cell lines. 76/59 showed little binding to American Burkitt's lymphoma but it did bind to cord blood and adult B cells. 80/97, in contrast, bound well to all cell lines tested, including African Burkitt's lymphoma-, myeloma-, T cell-, and lung epithelial carcinoma-derived cell lines.

Using quantitative radioimmunoassays at saturating conditions of protein A and antibody, it was demonstrated that in comparison to K562 cells, both peripheral blood lymphocytes and polymorphonuclear cells contained variably low quantities of antigens recognized by these two antibodies, but that the kinetics of binding to K562 and blood cells were similar. These results were consistent with a change in number as opposed to conformation of antigens on differentiation as compared to immature cells.

The molecular weights and some chemical characteristics of the antigens were determined using immunoprecipitation with Staphylococcus A. 76/59 recognized a doublet of approximately 33,000 molecular weight, while 80/97 recognized a distinctly different protein of lower molecular weight, about 27,000. Both antigens also label in the presence of ^{3}H-glucosamine, indicating the presence of sialic acid residues in their antigens.

The functional significance of the antigens remains uncertain. From previous work, it was known that 76/59 in the presence of complement inhibited CFU-C derived colony formation of human bone marrow cells. Using the fluorescent activated cell sorter, sterile sorting was performed. The total number of myeloid progenitors in normal human bone marrow was contained in the 76/59 antibody positive population. However, a substantial proportion of normal bone marrow cells stained with 76/59,

probably corresponding to early myeloid progenitors beyond the CFU-C stage. In a separate experiment, the ability of monoclonal antibodies to substitute for growth factors was tested using the ability of K562 cells to form colonies in semi-solid matrix. Both hybridoma supernatants and affinity purified immunoglobulins derived from ascites fluids were tested for their ability to stimulate colony formation in the absence of fetal calf serum, the usual source of growth factors. In fact, it was found that 76/59 IgG, purified by absorption to protein A sepharose replaced fetal calf serum as a stimulator of colony formation. These results suggest that a cell surface membrane protein related to or identical with a growth factor receptor was recognized by 76/59.

Proposed Course of the Project:

76/59 will be tested for its ability to bind to the putative growth factor receptor on human bone marrow cells. The antibody, which is available in large quantities, will be used to purify its membrane constituent. It may prove possible to use 76/59 in combination with other antibodies in the purification of hematopoietic cells or in identification of stem cells in bone marrow specimens.

Publications:

1. Young, N.S.: Aplastic Anemia: Research Themes and Clinical Issues. In Progress in Hematology, Vol XII (Ed.) Elmer B. Brown, Publisher - Grune & Stratton, Inc. 1981.

2. Young, N.S. and Hwang-Chen, Sheam-Pey: Anti-K562 cell monoclonal antibodies recognize hematopoietic progenitors. Proc. Natl. Acad. Sci. USA, Vol 78: 7073-7077, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 02310 02 CHB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Characterization of the Gene for Human Dihydrofolate Reductase in Normal Cells and Cells resistant to Methotrexate

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI -	M. J. Chen	Senior Staff Fellow	CHB NHLBI
Other -	Takashi Shimada	Visiting Fellow	CHB NHLBI
	Austine Davis Moulton	Research Assistant	CHB NHLBI
	Maria Harrison	Electron Microcoscopist	CHB NHLBI
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COOPERATING UNITS (if any)

None

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Three groups of recombinant bacteriophage containing coding sequences for dihydrofolate reductase (DHFR) were isolated from two human genomic DNA fragment libraries. One clone (λhDHFR-1) contains three coding blocks (exons) which encode the C-terminal half of human DHFR. The other two groups of recombinant each contain an intronless DHFR coding sequence. One recombinant (λhDHFR-ψ_2) includes the entire coding sequence; the presence of several in phase termination codons in all three reading frames indicate that DHFR-ψ_2 is a pseudogene. The overall sequence homology of hDHFR-ψ_2 to the available portions of the normal human DHFR coding sequences is 93%. On the other hand, the other intronless gene, (hDHFR-ψ_1) has an open reading frame and is virtually identical to the coding sequence of the normal gene with introns. The DNA sequence homology among the 3 genes extends 2.9 kilobases beyond the end of the coding sequences. Both intronless genes have an A-rich tract at the 3' end of the sequence homology. The lack of introns and the presence of A-rich tracts at the 3' ends of intronless genes suggest that these genes are derived from processed RNA intermediates. A short DNA sequence, 60 nucleotides 5' to the ATG initiation codon in hDHFR-ψ_2 is directly repeated immediately after the 3' A-rich tract providing evidence of DNA insertion.

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Objectives:

One objective of this study is to compare the mode of regulation of constitutively expressed genes to the mechanism of regulation of the genes which encode for products characteristic of the differentiated state. Constitutively expressed genes are generally crucial for cell proliferation and growth while specialized genes are most relevant to the development of the differentiated state. Dihydrofolate reductase (DHFR) was chosen for study because of its essential role in cell metabolism; it is required for the synthesis of deoxynucleotide precursors utilized for DNA synthesis.

The second objective of these studies is to characterize the mechanism of gene amplification. Methotrexate is a selective inhibitor of dihydrofolate reductase. Cultured human and rodent cells, grown in the presence of this agent, often become resistant to methotrexate. Frequently this is due to DHFR gene amplification which increases the level of DHFR mRNA and protein. Our purpose is to compare the structure and organization of functional DHFR genes in normal and methotrexate resistant cells and thereby to deduce certain features relevant to the mechanism of gene amplification. These studies are done in collaboration with Dr. Ken Cowan of the Medicine Branch in the NCI.

Methods:

1. Molecular cloning: Available human genomic DNA fragment libraries cloned into bacteriophage, were screened by the method of Benton and Davis using a cloned mouse DHFR cDNA as a probe. Several recombinants containing DHFR coding sequences have been identified, purified by rescreening, and characterized by restriction endonuclease mapping. To increase the probability of obtaining the intact human DHFR gene, a cosmid library consisting of fragments 35-40 kilobases in length, has been constructed in the standard cosmid cloning vector. This library will be screened with probes derived from already characterized human DHFR containing clones to increase the probability of obtaining the 5' end of the human DHFR gene.

2. Heteroduplex analysis: Regions of sequence homology in cloned DNA fragments are defined by electron microscopy. The purified fragments are denatured, allowing to reanneal, shadowed, and the resulting grids are studied at high amplification.

3. DNA sequencing: DNA fragments derived from recombinant bacteriophage containing DHFR gene sequences are subcloned into the plasmid vector, pBR322. Appropriate fragments are selected for sequencing. These are end labelled and a family of DNA molecules differing in length by single nucleotides is generated by the standard Maxam and Gilbert degradation procedure. Resolution of these on thin polyacrylamide gels in urea, followed by radioautography, allows the sequence to be determined. Alternatively fragments are cloned into the single stranded bacteriophage, M13, and dideoxynucleotides are used to generate families of DNA molecules to be resolved on polyacrylamide gels.

4. Analysis of mRNA: Human DHFR mRNA is extracted from cultured cells and the precise site(s) at which transcription is initiated and terminated is determined using S_1 nuclease mapping techniques.

Major Findings:

1. One half of the normal human DHFR locus has been characterized by molecular cloning, restriction endonuclease mapping and DNA sequencing of the coding portions.

2. Two intronless human DHFR genes have been identified. Features which suggest that these genes are derived from processed RNA intermediates are the removal of intron sequences precisely by the rules of RNA splicing and the presence of A-rich tracts at the 3' end of the homology to the normal gene. Directly repeated DNA sequences at the boundaries of one intronless DHFR gene provide evidence of its insertion into the chromosome.

3. One of the intronless DHFR genes, which we have characterized, is virtually identical in sequence to the corresponding portions of the functional DHFR. Only one nucleotide in 800 was found to be different. This data provides evidence of the recent evolutionary origin of this gene and furthermore provides proof that the process of reverse transcription required to convert RNA molecules back into DNA in eukaryotic cells occurs with extremely high fidelity. Preliminary data suggest that this "perfect" intronless gene is found in most but not all human DNA samples suggesting that it arose following the development of the human species.

4. One amplified DHFR line has been studied by restriction endonuclease analysis and Southern blotting of its DNA. Amplification of the DHFR gene with introns was demonstrated. In contrast, the two intronless genes were not amplified in the methotrexate resistant cell line. This data provides evidence that these genes are not functional and furthermore that they are not part of the amplification unit containing the functional DHFR allele.

Significance to Biomedical Research and the Program in the Institute:

This project is designed to increase our understanding of transcriptional signals which operate at the DNA sequence level in modulating gene expression. The phenomenon of gene amplification, if understood and applied, might have important implications for genetic engineering of eukaryotic cells. Furthermore the DHFR gene is one of the few available potential dominant selectable markers for use in gene transfer into eukaryotic cells.

Proposed Course of the Project:

Our immediate objective is to isolate the 5' end of the functional human DHFR gene from the cosmid library of human DNA fragments which has been constructed. Analogous cosmid libraries will also be constructed using DNA from cell lines in which the DHFR gene has been amplified. Comparison of the structure of the gene will be done by standard restriction endonuclease mapping and heteroduplex techniques. Transcriptional signals will be characterized by DNA sequencing and by functional studies in eukaryotic expression systems. Furthermore, the intact human DHFR gene will be used as a dominant selectable marker in an effort to introduce DNA into hematopoietic stem and progenitor cells *in vitro* and *in vivo*.

Publications:

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02311 01 CHB

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Development of a General Method for Gene Cloning Using Eukaryotic Expression Vectors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI	George Keller	Staff Fellow	CHB	NHLBI
	Guy Weinberg	Medical Staff Fellow	CHB	NHLBI
	Keith Humphries	Visiting Associate	CHB	NHLBI
Other:	Neal S. Young	Senior Investigator	CHB	NHLBI
	Arthur W. Nienhuis	Chief, Branch	CHB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH-Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to develop a set of techniques which may allow one to clone specific eukaryotic genes using only antibodies as the screening probe. Our strategy is to construct a human genomic DNA library using a unique cosmid expression vector, transfer these cloned sequences to cultured eukaryotic cells, detect those cell colonies producing the protein of interest and finally, rescuing the plasmid, with its cloned sequence, from the expanded colony. We are currently constructing a human genomic library using a cosmid vector containing the bovine papilloma virus genome. Experiments are underway to optimize the transformation of cultured eukaryotic cells with plasmid DNA as well as experiments to optimize the detection of heterologous antigens in individual cultured cells. When these techniques are available, they should permit the cloning of eukaryotic genes which would otherwise be inaccessible.

450

Objective

The objective of this project is to devise a system for eukaryotic gene cloning which will allow us to detect and isolate specific genes, using antibodies directed against the gene product. Antibodies against several cell proteins of interest in hematopoiesis are available and it should be possible to clone the genes for these proteins, given such a cloning system. These genes are not amenable to cloning by more traditional techniques (i.e., cross-screening of cDNA libraries) due to the small amount of protein and hence, mRNA that they produce.

In order to detect cloned genes with an antibody, it will be necessary to clone the entire human genome (or a specific portion of it) in a vector which will assure expression of the gene. Also, the cloned DNA will have to be transferred to a eukaryotic cell line in order for the expression vector to function. Next, 10,000 to 500,000 transformed cell colonies (depending on the source of DNA) will have to be screened with antibody, to detect colonies producing the protein of interest. Finally, positive colonies will be expanded, and the plasmid DNA rescued, by standard techniques.

Methods:

1. Cloning of Human Genomic DNA in an Expression Vector: We have constructed an expression vector consisting of 10 kb bovine pappiloma virus genomic fragment, a 1.5 kb λDNA fragment containing the "COS" region and a 2 kb pBR322 fragment containing the ampicillin resistance gene. Human DNA, partially digested with Ava I, will be ligated into the unique Sal I site of the vector and packaged into λ phage heads. These hybrid phage will then be used to establish an "expression library" in E. coli. Approximately 500,000 colonies will be needed for a "complete" library, using this approach. The choice of the BPV genome for our expression vector was made for 3 reasons.

1) Plasmids containing the BPV genome can transform certain rodent cell lines, providing a selection for those cells which take up DNA.

2) Such plasmids also replicate in the cells and remain episomal; enhancing our ability to detect any protein products and rescue the plasmid from the cultured cells.

3) The BPV DNA contains "activating sequences" which can enhance the transcription of genes cloned into the vector.

The λ COS fragment permits the cloning of large DNA fragments (up to 41 kb) and efficient transformation of E. coli with the packaged DNA.

2. Transfer of Library Sequences to Cultured Cells: The transformation of cultured cells with the genomic DNA cloned in E.coli must be an efficient process. The techniques of protoplast fusion and DEAE-dextran mediated transfer are being investigated because these techniques are more efficient at transferring DNA into cells than the widely used $CaPO_4$ precipitation technique. We are striving for an efficiency of about 6,000 transformants per microgram of DNA (starting with 2×10^5 cultured cells). At this transformation frequency, we can realistically screen the large number of colonies that would be required to find the gene of interest. Subpools of the E. coli library will be used to transform multiple dishes of cultured cells, which will then be screened as described below.

3. Detection of the Gene Product of Interest: Two methods will be investigated for the immunological detection of cells of colonies expressing a particular human gene. Cells can be removed from the dishes, incubated with the apropriate antibody, followed by fluorescent tag and sorted on a fluorescence-activated cell sorter (FACS). Positive cells can be collected and grown up for plasmid rescue. The other method involves replica plating of colonies onto nitrocellulose, lysis and incubation with first antibody, second antibody and ^{125}I-protein A. Positive colonies would be identified on the original dish, picked and grown up for plasmid rescue. Each method has distinct advantages and disadvantages for screening large numbers of cells or colonies.

4. Plasmid Rescue from Cultured Cells: Since the plasmids will remain episomal in the cultured cells, they can be recovered by any method for isolating low molecular weight DNA (i.e., Hirt extract). Plasmids can then be subcloned in *E. coli* in order to further purify the sequence of interest (in the case of more than one plasmid per cultured cell), and to provide a stable source of plasmid.

Proposed Course of the Project:

All of the above techniques will be pursued simultaneously in an effort to create an interlocking set of methods and a new approach to gene cloning. Upon reaching that goal, a specific gene of interest in erythropoiesis will be cloned, to demonstrate the usefulness of the methodology. A good candidate is the gene for the transferrin receptor, since antibody is readily available and since transferrin uptake is critical in erythroid development.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02312 01 CHB

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Effect of 5-Azacytidine on Fetal Hemoglobin Synthesis in Patients with Severe Thalassemia and Sickle Cell Anemia.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI - Timothy Ley		CHB NHLBI
Other - Arthur W. Nienhuis	Branch Chief	CHB NHLBI
Joseph DeSimone	Staff Geneticist**	
Paul Heller	Chief of Hematology**	
Patricia Griffith	Clinical Nurse Specialist	CHB NHLBI
Neal S. Young	Senior Investigator	CHB NHLBI
R. Keith Humphries	Visiting Associate	CHB NHLBI

COOPERATING UNITS (if any)

** West Side V.A. Medical Center, Chicago, Ill.

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

TOTAL MANYEARS: 1	PROFESSIONAL: 1	OTHER:

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Reactivation of the fetal γ globin genes might compensate for deficient β globin synthesis in patients with severe β thalassemia, thereby making erythropoiesis more effective and reducing the need for transfusions. 5-Azacytidine (5-AzaC) has been shown to stimulate Hb F production in anemic baboons. This protocol was designed to determine whether 5-Aza would have a similar effect on fetal hemoglobin production in humans. Patients are given a continuous intravenous infusion of 5-AzaC at a dose of 2 mg/kg/day; this dose was found to cause minimal toxicity in patients with hematologic malignancies. Sequential bone marrow aspirates were analyzed for globin protein synthetic rates, mRNA concentrations, and the methylation status of DNA in the globin gene region. Two patients have been studied thus far. The first is a patient with β thalassemia and severe iron overload. γ globin synthesis increased seven-fold in this patient, normalizing the previously unbalanced globin synthetic ratio, the absolute reticulocyte count and hemoglobin concentration therefore increased. Hypomethylation of DNA near the ε and γ globin genes was directly demonstrated. The γ globin genes were expressed at a level of 7,000 copies per cell, in contrast to 15 copies of ε globin mRNA per cell. A second patient with sickle cell

disease has been treated; preliminary results suggest a similar augmentation of Hb F production and markedly decreased "sickling" after therapy.

Objectives:

This study was designed to determine whether 5-AzaC would augment γ globin synthesis in human subjects, and to further understand the mechanisms of globin gene regulation. The γ globin genes are relatively hypomethylated in tissues where they are highly expressed, and completely methylated in normal adult bone marrow. 5-AzaC is capable of activating genes by causing undermethylation of newly synthesized DNA. This drug was administered in order to cause hypomethylation of DNA near the γ genes, and hopefully, to activate γ globin synthesis. These studies therefore represent an effort to manipulate globin gene expression in a therapeutically useful way in man.

Methods:

Patients who participate in the 5-AzaC trial will have severe β thalassemia (or sickle cell disease) associated with severe transfusional hemochromatosis and a poor long-term prognosis. Before the 5-AzaC infusion, complete laboratory evaluation and two control bone marrows are performed. 5-AzaC is then infused intravenously for seven days at a rate of 2 mg/kg/day. Daily laboratory studies are performed to monitor for signs of toxicity. Serial bone marrow examinations are performed weekly for three weeks after the infusion. The bone marrow samples are radiolabeled to identify newly synthesized proteins, and RNA and DNA are purified. The following molecular studies are performed:

1. 2×10^7 bone marrow cells are labeled with [$^{C}14$]leucine in leucine-free IMEM. Lysates of these incubations are analyzed on Triton X - urea gels, and globin biosynthetic ratios are determined by densitometry tracings of the fluorograms.

2. Bone marrow cells are lysed in guanidinium hydrochloride and sarcosyl, and then processed to obtain RNA and DNA. The RNA is analyzed using RNA spot blotting and S_1 nuclease analysis. The DNA is analyzed by restriction endonuclease analysis and Southern blotting techniques in order to determine the frequency of methylated cytosine residues in various regions of total bone marrow DNA.

3. 1×10^7 bone marrow cells are being cultured for a variety of studies. These include an assessment of the relative proportions of BFU-E and CFU-E, evaluation of erythropoietin sensitivity, and pulse labeling of colonies with [^{3}H]leucine followed by isoelectric focusing in order to determine the globin biosynthetic pattern in erythroid colonies.

Major Findings:

In the first patient treated, 5-AzaC administration led to a 7-fold increase in γ globin synthesis, temporarily normalizing the patient's unbalanced globin chain synthetic ratio. Erythropoiesis became more effective, leading to an increase in the absolute reticulocyte count ($5000/mm^3$ -> $22,000/mm^3$) and hemoglobin concentration (8.0 gm/dl to 10.8 gm/dl). The patient's hemoglobin remained at a level of >9 gms/dl for 40 days; he did not require transfusions during this period. Hypomethylation of bone marrow DNA near both the ε and γ genes was demonstrated. However, at the time of peak drug effect, 7,000 γ mRNA molecules were present per bone marrow cell, in contrast to only 15 ε-globin mRNA molecules per cell. α globin gene expression was unchanged with treatment and β globin mRNA concentration decreased slightly after the infusion. Expression of the human α2[I] collagen gene was unchanged in bone marrow following 5-AzaC treatment.

Patients with transfusion dependent β thalassemia or sickle cell disease could benefit immensely from augmented γ globin chain synthesis and increased formation of Hb F. The ability to chronically increase hemoglobin F synthesis might reduce or eliminate the need for transfusions, allowing iron stores to be reduced by chelation or phlebotomy. Studies designed to determine the mechanism of 5-AzaC action will yield new information regarding the mechanism of globin gene regulation.

Proposed Course of the Project:

We plan to evaluate two to four more β^+ thalassemic patients with 5-AzaC infusions before terminating the first phase of this project. These studies are designed to assure that 5-AzaC is capable of causing increased γ globin synthesis in several patients who have this disorder; this will establish that this technique may be generally effective. If most patients do respond, we will attempt to develop a regimen that would allow a continued high level of γ globin gene synthesis. To achieve this objective, chronic admiistration of 5-AzaC to primate (rhesus) alone or in combination with other cytotoxic drugs will be utilized to develop a maximally effective and minimally toxic regimen. Ultimately we hope to develop a regimen which will either temporarily or permanently eliminate the need for blood transfusion in selected patients with severe beta thalassemia. Agressive chelation therapy to remove excess iron will be given to such patients to reverse the adverse effect of iron overload.

Publications:

None

ANNUAL REPORT OF THE
LABORATORY OF EXPERIMENTAL ATHEROSCLEROSIS
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1981 through September 30, 1982

Localized atherosclerotic thickenings of arterial vessel walls contribute significantly to human morbidity because of their role in the pathogenesis of heart attack and stroke. These atherosclerotic lesions are composed of cells and lipid-rich particles which together comprise the predominant mass of lesions. For this reason, our research program is focussing on characterizing and determining the origin of lipid-rich particles and cells within atherosclerotic lesions.

Histochemical characterization of lipid-rich particles in human and experimentally induced atherosclerotic lesions has been carried out using the fluorescent probe filipin to detect free cholesterol and the lipid-soluble dye oil red O to detect esterified cholesterol. Filipin staining of cholesterol ester has been accomplished by enzymatically (using cholesterol esterase) converting cholesterol ester to free cholesterol before staining. Using these staining methods, it has been determined that cholesterol ester accumulates intracellularly within lipid droplets and extracellularly in smaller particles apparently distinct and unrelated to intracellular lipid droplets. Some cells which are stained with oil red O are not stained with filipin even after treatment of tissue sections with cholesterol esterase. This suggests that not all lipid-containing cells in atherosclerotic lesions accumulate cholesterol.

We have also made new observations concerning the location and form of accumulated free cholesterol within atherosclerotic lesions. Free cholesterol accumulates separately from cholesterol ester and is located predominantly in the extracellular space in association with three structures: free cholesterol-rich particles, crystals, and calcium granules. Rare cells contain exclusively free cholesterol. The fact that free and esterified cholesterol accumulate separately in different structural forms suggests that a variety of metabolic pathways mediate cholesterol accumulation in atherosclerotic lesions. However, the fact that similar cholesterol-rich structures are present in atherosclerotic lesions of humans and swine, and in experimentally induced lesions of rats, rabbits, and monkeys fed a high cholesterol diet, suggests that the general mechanism of cholesterol deposition is very similar. This appears to be so even though the histological appearance of lesions varies considerably among these species. Experimental systems will now be developed with the objective of generating cholesterol-rich particles in vitro.

A new project has been initiated this year to isolate and characterize the cholesterol-rich particles described above. Intimas dissected from human atherosclerotic vessels have been homogenized to release lipid-rich particles. Using differential centrifugation, it has been possible to obtain a cholesterol-rich fraction which contains particles similar to those observed microscopically in tissue sections. Preliminary results indicate that four subpopulations of particles exist which vary in size and in cholesterol ester and free cholesterol content. Work is proceeding to separate these cholesterol-rich particles into chemically and morphologically defined populations using gel filtration chromatography and fluorescence-activated particle sorting of filipin-

stained cholesterol-rich particles.

Cells are an important component of atherosclerotic lesions. Besides contributing to the mass effect of lesions, they participate in the accumulation of cholesterol. We have initiated studies to characterize cells isolated from atherosclerotic lesions using flow cytometry and sorting. Flow cytomery is a technique in which measurements of light scatter (indicating cell size and structure) and fluorescence emission are simultaneously carried out on fluorescently stained cells as they flow in suspension one at a time past a laser. Specific cell populations may be purified by the process of sorting. To prepare single cell suspensions for use in flow cytometry, we have developed a method of mild homogenization for isolating cells from formalin-fixed autopsy vascular tissue. Viable animal vascular tissue has been dissociated using enzymatic digestion. The potential for recognizing and sorting vascular nucleated cells stained with a DNA-specific fluorescent dye has been demonstrated. Our attempt to quantify circulating endothelial cells using flow cytometry has been hindered by the lack of a specific endothelial cell marker. Continuation of this study will depend on future developments concerning availability of antisera directed against endothelial cell determinants.

In conclusion, efforts in our lab are directed at characterizing and defining the mechanisms by which cholesterol-rich particles and cellular elements contribute to the development of atherosclerotic lesions. We have been successful in defining new morphological forms in which cholesterol accumulates within lesions. Our finding that marked compartmentalization and diversity of cholesterol-rich structures occurs within lesions suggests that the metabolism and cell biology of atherosclerotic lesions are more complex than previously recognized.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 02823-03 EA

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Localization of Cholesterol in Atherosclerotic Lesions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. S. Kruth	Senior Investigator	EA NHLBI

COOPERATING UNITS (if any)
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Pathological Anatomy Department, Clinical Center, NIH

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION
Vascular Physiology

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	1.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Free and esterified cholesterol have been localized in human and experimentally induced atherosclerotic lesions using the fluorescent cholesterol probe filipin and the lipid-soluble dye oil red O. It has been determined that cholesterol ester accumulates intracellularly within lipid droplets and extracellularly within smaller particles apparently unrelated to intracellular lipid droplets. Free cholesterol accumulates separately from cholesterol ester and is located predominantly in the extracellular space in association with three structures: free cholesterol-rich particles, crystals, and calcium granules. Rare cells contain exclusively free cholesterol. The fact that free and esterified cholesterol accumulate separately in many different structural forms suggests that a number of metabolic pathways mediate cholesterol accumulation in atherosclerotic lesions.

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Objective: To localize free and esterified cholesterol in human and experimentally induced atherosclerotic lesions.

Methods: Tissue sections prepared from human and experimentally induced atherosclerotic lesions have been stained with the fluorescent probe filipin and the lipid-soluble dye oil red O to localize free and esterified cholesterol respectively. Filipin staining of cholesterol ester has been accomplished by enzymatically converting esterified cholesterol to free cholesterol before staining. Atherosclerotic lesions have been induced in rats, rabbits, and monkeys by feeding high cholesterol diets.

Major Findings: Using filipin, free cholesterol has been shown to accumulate within human atherosclerotic lesions in association with three extracellular structures: free cholesterol-rich particles, crystals, and calcium granules. Free cholesterol-rich particles comprised a substantial amount of accumulated cholesterol in many lesions. These particles were also present in atherosclerotic lesions of rats, rabbits, and monkeys fed a high cholesterol diet. Rare cells containing exclusively free cholesterol were detected using filipin but were not stained using conventional lipid-soluble dyes such as oil red O. Oil red O did stain inclusions in many cells. Filipin staining of sections treated with cholesterol esterase confirmed that these inclusions were cholesterol ester-containing lipid droplets. Interestingly, not all inclusions which stained with oil red O contained cholesterol. This suggests the possibility that some cells contain lipid other than cholesterol.

Many extracellular cholesterol ester-rich particles appeared similar in size to intracellular cholesterol ester-containing lipid droplets when sections were stained with oil red O. However, this appears to be an artifact caused by oil red O-induced fusion of extracellular cholesterol ester-rich particles. Extracellular cholesterol ester-rich particles detected using filipin were uniform in size and smaller than accumulated intracellular lipid droplets. Similar to what we reported last year concerning spontaneous swine atherosclerosis, extracellular free and esterified cholesterol accumulated in human and experimentally induced atherosclerotic lesions in a mutually exclusive distribution.

Significance: Use of the cholesterol probe filipin to stain free and esterified cholesterol in atherosclerotic lesions has led to a number of new observations. Extracellular free cholesterol-rich particles (not detected with conventional fat stains) comprise a substantial amount of accumulated cholesterol in lesions. Extracellular free cholesterol-rich and cholesterol ester-rich particles and intracellular cholesterol ester-containing lipid droplets are consistently present in human lesions and in experimentally induced atherosclerotic lesions of swine, monkeys, rats, and rabbits. These findings suggest that although the histological appearance of lesions in these species is different, the mechanism of lipid accumulation appears to be similar.

The structural dissimilarity of intracellular cholesterol ester-containing lipid droplets and extracellular cholesterol ester-rich particles suggests that degenerating "foam cells" may not, as commonly thought, be the source of extra-

cellular cholesterol. The fact that cholesterol accumulates separately in free and esterified forms in association with multiple structures (intracellular lipid droplets and extracellular particles, crystals, and calcium deposits) suggests that a number of metabolic pathways mediate cholesterol accumulation in atherosclerotic lesions.

Proposed Course: A project has been initiated to isolate and further characterize cholesterol-rich particles present in atherosclerotic lesions (see report Z01 HL 02826-01 EA). In addition, experimental systems will be developed for the purpose of generating cholesterol-rich particles in vitro.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 02824-03 EA

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Flow Cytometric Analysis of Cells Isolated from Atherosclerotic Lesions
(Revised Title)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. E. Cupp	Staff Fellow	EA NHLBI
Others:	G. N. Thomaidis	Visiting Fellow	EA NHLBI
	H. S. Kruth	Senior Investigator	EA NHLBI

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SECTION
Vascular Physiology

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	.5	.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
Atherosclerotic thickenings of blood vessel walls occur in humans and animals. These thickenings are composed of cells and accumulated lipid. Because of the importance of cells in atherogenesis, cell suspensions prepared from human and animal atherosclerotic lesions are being characterized using a new technology, flow cytometry and sorting. Flow cytometry is a technique in which measurements of light scatter (indicating cell size and structure) and fluorescence emission are simultaneously carried out on fluorescently stained cells as they flow in suspension one at a time past a laser. Specified cell populations may be purified by causing droplets containing desired cells to be deflected into a collection reservoir. Dissociation of human and animal atherosclerotic vessels has been carried out using mild homogenization and enzymatic digestion respectively. Different cell types are present in homogenates prepared from lesions. Light scatter and fluorescence analyses of these dissociated cells are being carried out.

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PHS-6040
(Rev. 2-81)

Objectives: To characterize cell types and cholesterol-rich cells isolated from atherosclerotic lesions using flow cytometric light scatter and fluorescence analyses of stained cells.

Methods: Flow cytometry is a technique in which measurements of light scatter (indicating cell size and structure) and fluorescence emission are simultaneously carried out on fluorescently stained cells as they flow in suspension one at a time past a laser. Specific cell populations may be purified by causing droplets containing desired cells to be deflected into a collection reservoir. Analysis of large numbers of cells can be rapidly carried out using this technique (up to 5000 cells/sec) in contrast to static scanning methods such as fluorescence microscopy. A Becton-Dickinson FACS II flow cytometer-sorter is being used in these studies.

Cell preparations for these experiments must be single-cell suspensions and have been prepared in two ways. We have developed a method of mild homogenization for isolating cells from formalin-fixed human autopsy vascular tissue. Viable animal vascular tissue has been dissociated into single cell suspensions using enzymatic digestion. Cells have been stained with DNA-specific probes to identify nucleated cells. Filipin, a cholesterol-specific stain, will be used to study cholesterol-containing cells. Cell types in lesions will be evaluated on the basis of size and structure using 0° and 90° light scatter respectively. This technique is useful in identifying subpopulations of cells which would not ordinarily be resolved using only low angle light scatter.

Major Findings: A variety of cell types are present in homogenates prepared from human atherosclerotic lesions. The potential for recognizing and analyzing nucleated cells in a homogenate based on fluorescence emission of stained DNA has been demonstrated. Examination of sorted fractions with a fluorescence microscope confirmed that nucleated cells had been sorted.

Significance: Flow cytometry and sorting is a methodology which provides a unique approach for the study of atherosclerotic lesions.

Proposed Course: Characterization of cell populations isolated from atherosclerotic lesions will be carried out using light scatter and fluorescence analyses.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02826-01 EA

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Isolation and Characterization of Lipid-Rich Particles in Atherosclerotic Lesions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. N. Thomaidis	Visiting Fellow	EA NHLBI
Others:	J. E. Cupp	Staff Fellow	EA NHLBI
	H. S. Kruth	Senior Investigator	EA NHLBI

COOPERATING UNITS (if any)
Pathological Anatomy Department, Clinical Center, NIH

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION
Vascular Physiology

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	.5	.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Free cholesterol-rich and cholesterol ester-rich particles accumulate in atherosclerotic lesions. A lipid-rich fraction containing particles similar to those observed microscopically in histological preparations (see report Z01 HL 02823-03 EA) has been isolated from human atherosclerotic lesions. Chemical and morphological characterization of fractionated cholesterol-rich particles is being carried out and indicates that four subpopulations of particles exist, which vary in size and free and esterified cholesterol content. Results of these studies should help determine the origin of accumulated particles and possibly suggest means to mobilize these pathological deposits of cholesterol.

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PHS-6040
(Rev. 2-81)

Objectives: To isolate and chemically characterize free cholesterol-rich and cholesterol ester-rich particles which accumulate in atherosclerotic lesions.

Methods: Conventional centrifugation, column chromatography, electrophoresis, and ultrafiltration methods have been employed in the isolation of cholesterol-rich particles. In conjunction with chemical analysis, filipin, a fluorescent probe which binds to free cholesterol, has been used to microscopically monitor the purification of free cholesterol-rich particles. In addition, fluorescence and light scatter analysis of isolated particles has been carried out using a fluorescence-activated cell sorter.

Major Findings: Lipid-rich particles isolated from human atherosclerotic lesions consist of four different subpopulations. Fluorescence microscopic studies indicate that filipin stains two subpopulations of large and small particles. Microscopic studies of filipin-stained tissue sections prepared from atherosclerotic lesions suggest that these particles are located extracellularly. It has been possible to elute separately these particle subpopulations from a sephacryl-1000 gel filtration column, however, some particle aggregation was present. The other two subpopulations of large and small particles do not stain with filipin and are refractile when observed by phase microscopy. In tissue sections, the larger refractile inclusions appear intracellularly, whereas the smaller ones are located extracellularly. Preliminary chemical analysis suggests that refractile particles have a greater cholesterol ester to free cholesterol ratio than filipin-stained particle populations. Light scatter and fluorescence analysis of filipin-stained particles using a fluorescence-activated cell sorter revealed both fluorescent (free cholesterol-rich) and nonfluorescent (free cholesterol-poor) particles.

Significance: Histochemical studies indicate that free cholesterol and cholesterol ester accumulate in discrete intra- and extracellular loci within the atherosclerotic intima (see report Z01 HL 02823-03 EA). These loci contain particles of uniform sizes which can be stained with either filipin (stains free cholesterol) or oil red O (stains cholesterol ester). Determination of the chemical composition of these different cholesterol-rich particles should contribute to a better understanding of the origin of pathological cholesterol accumulation within atherosclerotic lesions.

Proposed Course: Work will continue in the fractionation and purification of chemically and morphologically defined cholesterol-rich particles isolated from human atherosclerotic lesions.

ANNUAL REPORT OF THE
HYPERTENSION-ENDOCRINE BRANCH
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1981 through September 30, 1982
Section of Experimental Therapeutics

This year the work of the Hypertension-Endocrine Branch has included studies of a number of different vasoactive systems: i.e., 1) sympathetic nervous system-catecholamines; 2) prostaglandins; 3) endogenous opiate peptides; and 4) arginine vasopressin. Studies of all these systems and of their interactions were performed to delineate the pathogenesis of hypertension and to develop better forms of therapy for this serious common disease.

We have reviewed over 70 published studies in which plasma levels of either norepinephrine (NE) or total catecholamines were measured in both normals and in patients with essential hypertension. While there was considerable variability in the results and less than half of the studies reported a significant difference between the catecholamine levels in the two groups, when each study served as single data points then there clearly was a higher level of plasma norepinephrine in patients with essential hypertension. This was especially true in young subjects matched for age. The review indicated that in normals plasma norepinephrine increased with age, while no such change was found in hypertensives. This finding was tested by analysis of data obtained previously at the Uniformed Services University for the Health Sciences. Across 129 normotensives, plasma NE increased significantly with age, but did not across 191 hypertensives, due to relatively high NE values among young hypertensives. When patients and controls less than 40 years old were considered, hypertensives showed significantly higher norepinephrine levels than the controls ($P < 0.01$). But above the age of 40, no significant hypertensive-normotensive difference was obtained. The data are consistent with increased sympathetic nervous system activity in the early stages of essential hypertension.

We have recently developed and validated assay techniques for measuring plasma catecholamines in our laboratory. We can now use these techniques for testing hypotheses about the role of the sympathetic nervous system in hypertension. The technique makes use of high pressure liquid chromatography and electrochemical detection to measure plasma levels of norepinephrine, epinephrine and dopamine in human subjects. We have measured arm arterial and venous catecholamines to assess the contribution of peripheral vascular sympathetic activity as reflected by arterial venous differences in norepinephrine as well as the presumed uptake of epinephrine by peripheral vessels as reflected by arterial venous differences in epinephrine. We have done this so far in 14 normotensive and 15 patients with essential hypertension. Hypertensives have shown higher arterial and venous norepinephrine levels than normotensives, as well as greater arterial venous differences in epinephrine. Hypertensives have also shown higher arterial and venous epinephrine levels and an accentuation of the arterial venous difference in epinephrine. These results suggests that accentuated peripheral vascular sympathetic activity occurs in essential hypertension, and that both abnormal sympathetic neural and sympathoadrenomedullary activity characterize a proportion of patients with essential hypertension.

Plasma norepinephrine levels represent the balance of synthesis and release, as well as reuptake and degradation. In patients with hypertension increased levels of plasma catecholamines could represent increased synthesis, decreased

reuptake or decreased metabolism. We have used both L-, and D-, norepinephrine as a means of studying norepinephrine kinetics in both healthy persons and patients with essential hypertension. C-14 labeled D norepinephrine, tritiated L-norepinephrine, and tritiated isoproterenol are infused together for 20 minutes, and blood samples taken during the infusion and up to 60 minutes afterwards. Norepinephrine and isoproterenol peaks are separated using liquid chromatography with electrochemical detection and the C-14 and tritium counted using liquid scintillation spectrometry. The ratio of C-14 to tritium in norepinephrine provides a measure of the stereo-selective components of norepinephrine disappearance, while the ratio of tritium in isoproterenol to that in norepinephrine provides a measure of neuronal norepinephrine uptake. So far 10 hypertensives and 12 normotensive subjects have been studied. Although supine, resting, and preinfusion venous plasma norepinephrine levels were significantly higher in the hypertensives, the hypertensive and normotensive groups did not differ significantly in the disappearance kinetics of L- or D-norepinephrine or isoproterenol. The overall increased resting plasma norepinephrine levels seen in essential hypertension therefore appear to derive from increased sympathetic neural activity, not diminished disappearance of plasma norepinephrine. Because isoproterenol is not taken up by nerve endings, the ratio of tritiated L-norepinephrine increased after the infusion ended. This increase was almost completely abolished by pretreatment with the neuronal norepinephrine uptake blocker, desipramine, in 5 subjects. Thus changes in the isoproterenol: norepinephrine ratio after simultaneous infusion of both provide a sensitive new index of neuronal norepinephrine uptake in man.

The synaptic cleft is the place where both endogenously released norepinephrine and exogenously infused norepinephrine meet to produce their pressor action. Both D-amphetamine and tyramine release norepinephrine from sympathetic neural storage vesicles. Therefore we determined the extent of agreement between the pressure response - indicative of the increment in synaptic cleft norepinephrine after D-amphetamine or tyramine - and the circulating norepinephrine response-indicating "spillover" of norepinephrine from the cleft into the general circulation. To do this, patients were infused with a single intravenous infusion of D-amphetamine over a 5 minute period or with graded bolus doses of tyramine to achieve an increment in systolic pressure of about 25 mm Hg. Both D-amphetamine and tyramine dramatically increase systolic blood pressure associated with increased plasma norepinephrine but unassociated with changes in plasma epinephrine or heart rate. The time course of pressure and norepinephrine responses to tyramine was much shorter than D-amphetamine. Change in systolic pressure per unit change in norepinephrine was similar with the two drugs, consistent with the hypothesis that both agents increased blood pressure by an increased synaptic cleft norepinephrine, and that circulating norepinephrine reflects "spillover" from the cleft into the general circulation.

We recently reviewed the literature on the effects of physical stress on patients with essential hypertension and noted that such patients appear to show excessive sympathetic responsiveness to physical stress. We next wondered whether patients with essential hypertension would show a similar excessive response to nonphysical stress. While a number of different procedures have been used in the past, we felt that undergoing wisdom tooth extraction is a real-life, acute emotional stress which was readily available to us at the Clinical Center. We have now studied 21 healthy control patients and found that the surgery was associated with a significantly increased heart rate (+ 25%), systolic blood pressure (+13%), and cardiac output (+ 34%) without a significant change in diastolic blood pressure. Plasma norepinephrine increased by 60% during the surgery in nonsedated patients. Diazepam sedation abolished the norepinephrine and epinephrine response

to the surgery, without significantly affecting the heart rate or systolic blood pressure responses. Receipt of epinephrine in the local anesthetic resulted in a 5-fold increase in mean plasma epinephrine 5 minutes after the injection, as well as increased cardiac output. The direct effect of epinephrine accounted for the cardiac output increase observed during the surgery. In nonsedated patients, epinephrine increased about 3-fold with the surgery. Diazepam appeared directly to decrease norepinephrine but not epinephrine levels. These results suggest the participation of the sympathetic nervous system in producing the circulatory responses to dental surgery. The elimination of sympathetic recruitment by diazepam-induced sedation, however, without concomitant reductions in heart rate or systolic blood pressure responses, suggest that other systems besides the sympathetic nervous system influence the circulatory response to this real-life stress. In contrast with isotonic exercise, which produces greater norepinephrine than epinephrine responses in healthy persons, the emotional stress of dental surgery produces greater epinephrine than norepinephrine responses. We now propose to continue these studies in patients with hypertension and to study normotensive children of hypertensive parents.

Pheochromocytomas are catecholamine-secreting tumors which can cause hypertension. They represent the unusual situation of surgically curable hypertension. We have been making use of our new techniques to measure plasma catecholamines and test them as useful diagnostic procedures for this uncommon tumor. Across 15 patients with surgery-proven pheochromocytoma and 41 patients with hypertension but without pheochromocytoma, the plasma norepinephrine distribution showed only slight overlap. Two patients with pheochromocytoma showed plasma norepinephrine levels within two standard deviations of the mean of the non-pheochromocytoma patients; the remainder showed plasma norepinephrine levels exceeding 2 SD's above the non-pheochromocytoma mean. These results suggest that exceedingly high norepinephrine levels-above 600 pg/ml in resting, supine patients with hypertension support the diagnosis of pheochromocytoma, but the levels less than about 600 pg/ml do not exclude the diagnosis.

The baroreflex is the most powerful, rapidly acting, circulatory, homeostatic reflex. Theoretically, hypertension can occur only when there is an abnormality in the function of this reflex mechanism. However, previous studies have disagreed about whether hypertensives show abnormal reflex sensitivity and there have been no comparisons of the various techniques used in these studies. We have therefore used three basic techniques, i.e., 1) neck cuff apparatus, especially designed to allow delivery of rapid changes in external neck pressure; 2) the Valsalva maneuver; and 3) injections of phenylephrine and nitroglycerin, to evaluate this baroreflex. These techniques have actually provided eight separate measures in the same subject of baroreflex sensitivity. The average intercorrelation among these measures in 30 subjects was statistically significant ($r = 0.36$, $P > 0.01$), but suggests that on average variance in one measure accounted for only about 13% of the variance in others. Standard deviations across subjects often were as large as the means, indicating important interindividual variability as well. Nevertheless, when age matched subgroups of 10 hypertensives and 7 normotensives were compared, baroreflex ranking averaged across measurement techniques were significantly lower in the hypertensives ($t = 3.27$, $P > 0.01$). Patients with essential hypertension therefore do appear to have diminished baroreflex sensitivity. We have recently tested the effects of clonidine therapy on baroreflex sensitivity in three hypertensive patients. Clonidine decreased resting venous plasma norepinephrine by over 70% in these patients, resting systolic and diastolic pressures decreased to the normotensive range, and the overshoot in systolic pressure after release of the Valsalva maneuver was markedly attenuated. All these findings are consistent with effective

therapeutic levels of Clonidine; however, no obvious change in baroreflex sensitivity appeared. These data so far are inconsistent with the hypothesis that Clondine's antihypertensive effect derives from accentuated baroreflex sensitivity. In all our studies we have noticed an inverse relationship between resting plasma norepinephrine and baroreflex-heart rate responsiveness. The former indicates sympathetic neural activity and the latter vagal responsiveness. Thus those hypertensives with accentuated resting sympathetic tone may also show depressed vagal tone. These findings would be consistent with an abnormality of central nervous system control of blood pressure in some patients, resulting in a pattern of vagal inhibition and increased sympathetic outflow. We are therefore studying baroreflex sensitivity before and after acute atropinization in order to determine if pressure hyperresponsiveness can be produced in healthy people when vagal reflex or heart rate changes are blocked.

In cooperation with Dr. Allen Harris at the Johns Hopkins School of Medicine, we have been able to demonstrate an association between sympathetic neural activity and blood pressure in baboons conditioned to raise their blood pressure on cue. In this study baboons undergo conditioning with reinforcement for increases in diastolic blood pressure during daily, 12 hour training sessions. The criterion blood pressure is increased over the course of several weeks using a shaping procedure. Blood pressure is measured continuously, and blood samples are obtained via chronically indwelling arterial and venous catheters. During the training sessions, systolic blood pressure increased significantly by 19 mm Hg (+ 15%), diastolic blood pressure 20 mm Hg (+ 30%), and heart rate 33 beats per minute (+ 33%) compared with presession values. However, the blood pressure elevations were completely reversible, demonstrating that chronically repeated pressor episodes in otherwise healthy individuals do not themselves cause sustained hypertension. During the session, norepinephrine increased significantly (+ 48%) as did epinephrine; while dopamine and renin activities were unchanged. Plasma norepinephrine correlated positively with systolic and diastolic pressure in all baboons tested in both baseline and training conditions.

Since plasma catecholamines are clearly elevated in a portion of patients with essential hypertension, and there appears to be no alteration in the metabolism of the catecholamines in these patients, then increased levels of norepinephrine should result in increased levels of total peripheral resistance. It would thus be important to evaluate cardiac output and total peripheral resistance in the same patients in whom we are measuring plasma catecholamines. This has not been done in the past because of the problem of making these measurements by noninvasive techniques. The best approach to this would appear to be the use of impedance cardiography. We are therefore determining the validity of impedance cardiography as a noninvasive measure of cardiac output and stroke volume in man by comparison with results obtained using the Swan-Ganz thermodilution technique. Across six patients tested so far, cardiac outputs determined by the two methods have agreed remarkably well-correlation coefficient being more than 0.9. We plan to evaluate four more patients using both techniques, and if the agreement across subjects continues to be excellent we will then introduce manipulations designed to change peripheral resistance or cardiac output to determine the agreement between the techniques within subjects. We feel that impedance cardiography offers a unique means of determining cardiac output and stroke volume and thus total peripheral resistance noninvasively in our ambulatory hypertensive and normotensive subjects.

Both prostaglandins and acetylcholine cause an increase in salt and water excretion when injected into the renal artery of the dog. Recently we have noted

that dogs pretreated with indomethacin showed only a transient diuresis and natriuresis to acetylcholine, whereas control dogs had a sustained diuresis and natriuresis. We therefore examined the effects of renal arterial infusions of prostaglandins E_2 and $F_{2\alpha}$ on the diuretic and natriuretic response to acetylcholine in indomethacin-treated dogs. Renal arterial infusion of acetylcholine produced a natriuresis and diuresis and an increase in renal plasma flow without a change in glomerular filtration rate or renin secretion rate. In dogs pretreated with indomethacin, renal arterial infusion of acetylcholine produced an increase, then a decrease in urinary flow and sodium excretion that was accompanied by a progressive fall in glomerular filtration rate and renal plasma flow and a progressive increase in renin secretion rate. Renal arterial infusion of PGE_2 but not of $PGF_{2\alpha}$ before and during the infusion of the acetylcholine restored the diuretic and natriuretic response to acetylcholine in indomethacin treated dogs. These data suggest that the normal diuretic and natriuretic response to acetylcholine requires a normal synthesis of prostaglandins, probably PGE_2.

There is considerable debate about whether endogenous opiate peptides are involved in the regulation of blood pressure and if they may be pathogenetically involved in essential hypertension. Measurements of plasma opiate levels have been done with great difficulty and lack validity. We have performed two experiments in an attempt to evaluate the role of these substances in hypertension. In the first study, patients with uncomplicated mild to moderate hypertension were treated with either clonidine or clonidine plus a diuretic to bring about control of their blood pressure. Subjects were then given increasing doses of naloxone, an opiate antagonist, before and during treatment with clonidine. Acute naloxone injection failed to produce any substantial effect on blood pressure in patients chronically treated with clonidine. In the second experiment, the drug Captopril was tested in renal-hypertensive rats. Captopril, an angiotension I converting enzyme inhibitor, is capable of lowering blood pressure in situations where the renin-angiotensin system is not responsible for blood maintenance. Angiotensin converting enzyme also acts as an enkephalinase in the inaction of opiate peptides. We therefore hypothesized that Captopril might lower blood pressure by prolonging the action of endogenous opiates. Male Sprague-Dawley rats were made hypertensive by placing a silver clip on one kidney and allowed to recover. Four to seven weeks later when the animals were clearly hypertensive they were given Captopril either before or after therapy with naloxone. The dose of Captopril used (2 ml/kg) lowered mean blood pressure by 28 mm Hg and was sufficient to block the action of 30 μg of Angiotension I administered intravenously. Naloxone (2 ml/kg) did not cause a significant change in the basal blood pressure of the rat and did not reverse the antihypertensive effects of Captopril. The dose of naloxone used, however, was capable of blocking the effects of large doses of either morphine sulfate or leucine-enkephalin. Thus it would appear that in these models of hypertension opiate peptides are not involved in the pathogenesis of hypertension or in the action of these two types of hypertensive drug.

There is considerable indirect evidence that a pituitary factor other than ACTH participates in the regulation of steroid production in the adrenal cortex. Recently it has been shown that a large precursor peptide is present in the pituitary gland which contains the amino acid sequence for ACTH, the lipotropines and the endorphins. β-endophin is the most potent endorphin with regard to its opiate activity. Therefore we examined the effects of β-endorphin and of several of its N- or C- terminal fragments on steroidogenesis in the in situ perfused adrenal gland of hypophysectomized dogs. We found that β-endorphin selectively stimulates the secretion of aldosterone without affecting that of cortisol, with a potency similar to that of ACTH. Met-enkephalin, α-endorphin and γ-endorphin had no effect on steroid production. This indicates that the active core of β-endorphin resides in the C-terminal portion of the molecule. The results suggest that β-endorphin may play a role in the regulation of adrenal steroidogenesis. 469

Hypokalemia, polyuria, hypernatrenia and overproduction of renal prostaglandins characterize prolonged treatment of dogs with desoxycorticosterone. We therefore performed studies in dogs that indicate that the hypernatremia is associated with an increase in plasma arginine vasopressin, despite a tendency for the osmotic threshold for vasopressin release to increase. The polyuria thus develops despite increases in plasma vasopressin and is unaffected by a further increase in plasma vasopressin produced by treatment with vasopressin. Treatment with indomethacin which corrects the overproduction of renal prostaglandins restores renal responsiveness to vasopressin and thereby corrects the polyuria, hypernatremia and increased secretion of vasopressin. These findings suggest that in disorders such as primary aldosteronism, hypokalemia, hypernatremia and polyuria, may be associated with an increase in circulating vasopressin and this may be a factor in the hypertension present in these syndromes. To evaluate the role of arginine vasopression further, we are currently evaluating the mechanism of blood pressure response induced by various osmotic and nonosmotic stimuli to arginine vasopressin in awake, unrestrained, bilaterally nephrectomized rats. Hypertonic saline administered to the Long Evans strain of rats induced a significant increase in mean blood pressure which was not observed in the Brattleboro strain of rats. The latter strain is used because they have a congenital absence of arginine vasopressin. While results are very preliminary there appears to be a dependence on the presence of arginine vasopressin for hypertension to develop. In addition, Naloxone, an opiate antagonist, had no effect on the rise in blood pressure in the Long Evans rats. Further studies are underway to characterize more completely the role of vasopressin in this model of acute hypertension.

Patients with Bartter's syndrome (hypokalemic alkalosis, hyper-reninemia, aldosteronism, increased adrenergic nervous system function, hyperprostaglandinuria, normal blood pressure) have a defect in platelet aggregation. The cause of this defect is not known. Plasma concentrations of cyclic AMP are high in Bartter's syndrome. It has been proposed that overproduction of a stable prostaglandin related to prostacyclin, the most potent anti-aggregatory substance, and a stimulator of cyclic AMP, could be the responsible factor. However, plasma concentrations of one of the stable active metabolites of prostacyclin, 6-keto-prostaglandin E_1, has been shown to be normal. We have therefore evaluated the regulation of cyclic AMP production by platelets from patients with Bartter's syndrome and from patients with familial hypokalemia. The results show that basal, PGE_1-stimulated, and PGE_1 stimulated, norepinephrine-inhibited cyclic AMP production by platelets in these disorders are subnormal. Treatment with indomethacin, an inhibitor of prostaglandin synthesis, did not correct the defect of cyclic AMP production. These results suggest that platelet cyclic AMP production in patients with Bartter's syndrome and in other patients with hypokalemia is impaired despite an overproduction of antiaggregatory prostacyclin.

Thyrotropin releasing hormone (TRH) is a tripeptide that has many physiologic functions in addition to that of regulating pituitary thyrotropin secretion. Among these effects is its ability to act in vivo as a partial antagonist of opiates and neurotensin. Recently it has also been shown that TRH is more effective than Naloxone in improving cardiovascular function and survival in experimentally induced endotoxic and hypovolemic shock as well as improving neurologic recovery after experimental spinal trauma. We therefore tested the effect of TRH on the hypotensive response to Captopril in the two kidney, one clip model of renal hypertension. In 13 consecutive animals TRH reversed the hypotension produced by Captopril. These results show the profound cardiovascular effects of TRH and suggest that other peptides may be very important in blood pressure regulation and that these peptides need not be classical opiate peptides. To show the complexity

of interactions involved, we have also recently studied the action of a serotonin uptake blocker, CGP-6085. It has significant hypotensive action and it has been proposed that the drug acts by blocking serotonin uptake in the brain. In spontaneously hypertensive rats, the antihypertensive effect of CGP-6085 could be blocked by Naloxone, and could be prevented by pretreatment with the inhibitor of serotonin biosynthesis, parachlorophenylalanine. These results suggest that serotonin is involved in the hypotensive action of CGP-6085, that a receptor blocked by Naloxone participates in its mechanism of action. Certain gut and brain peptides have central effects after systemic injection. However, the mechanism remains unexplained. In at least two instances, i.e., angiotensin II-induced-drinking, and cholecystokinin-induced-satiety, afferent abdominal vagal fibers seem to be involved in the transmission of peripherally-originated signals to the central nervous system. There have been reports of dramatic reversals by Naloxone of cardiovascular consequences of hypovolemic, endotoxic, and spinal shock models. It is not known if circulating opioid peptides are involved in the shock process and if peripheral afferent fibers are involved in the transmission of certain noxious stimuli to the central nervous system during shock. We therefore tested survival in male Sprague-Dawley rats to endotoxic shock before and after peripheral vagotomy and with and without treatment with either atropine or atropine methylnitrate, a drug which does not enter the brain. There were no significant differences between vagotomized and sham -operated rats in systolic,diastolic, or mean arterial pressure during the endotoxin-induced hypotension. In addition, mortality was not significantly changed by vagotomy and both atropine and atropine methylnitrate offered no protection to endotoxin shock. These results indicate that whatever peripheral signals are elicited by endotoxin that are involved in the central mediated hypotensive response, they are not conveyed to the central nervous system by vagal abdominal afferent fibers. Much more work is necessary to integrate all these findings into a meaningful explanation of the pathogenesis of human hypertension.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01929-03 HE

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

The Sympathetic Nervous System and Stress

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

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LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.20	0.20	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are measuring cardiovascular (heart rate, blood pressure, and cardiac output), biochemical (plasma norephinephrine, epinephrine, growth hormone, cortisol, cholesterol, triglyceride, and lipoprotein), and psychological (pain, anxiety) responses to wisdom tooth extraction in patients with essential hypertension and in normotensive controls with and without a family history of hypertension.

PHS-6040
(Rev. 2-81)

Objectives: Undergoing wisdom tooth extractions is a real-life, acute emotional stress. By measuring plasma norepinephrine levels--reflecting sympathetic neural activity--and cardiovascular variables, we hope to characterize the response to non-physical stress in normal persons and patients with essential hypertension, since essential hypertensives appear to show excessive sympathetic responsiveness to stress which may have pathophysiologic significance (1).

Methods: Blood samples drawn through indwelling intravenous needles have been assayed for the above biochemical parameters a few days prior to surgery, and during the pre-operative, operative, and post-operative conditions, for each of two sets of wisdom tooth extractions. For the first operation, patients were randomly assigned to receive or not to receive sedation with intravenous diazepam, and for the second, were assigned to receive or not to receive epinephrine with the local anesthetic. Impedance cardiography has been used as a non-invasive measure of cardiac output. Experienced pain and anxiety have been measured using standardized questionnaires. Plasma catecholamines were measured via high pressure lipid chromatography with electrochemical detection according to methods developed and validated in our laboratory.

Results and their significance: Across 21 healthypatients (2), the surgery was associated with significantly increased heart rate (25%), systolic blood pressure (13%), and cardiac output (34%) without a significant change in diastolic blood pressure. Plasma norepinephrine increased by 60% during the surgery in non-sedated patients. Diazepam sedation abolished the norepinephrine and epinephrine response to the surgery, without significantly affecting the heart rate or systolic pressure responses. Receipt of epinephrine with the local anesthetic resulted in a five-fold increase in mean plasma epinephrine five minutes after the injection, as well as increased cardiac output. The direct effect of epinephrine accounted for the cardiac output increase observed during the surgery. In non-sedated patients epinephrine increased about three-fold with the surgery. Diazepam appeared directly to decrease norepinephrine but not epinephrine levels.

The results suggest the participation of the sympathetic nervous system in producing the circulatory responses to dental surgery. The elimination of sympathetic recruitment by diazepam-induced sedation, however, without concomitant reductions in heart rate or systolic pressure responses, suggests that other systems besides the sympathetic nervous system influence the circulatory response to this real-life stress. In contrast with isotonic exercise, which produces greater norepinephrine than epinephrine responses in healthy persons, the emotional stress of dental surgery produces greater epinephrine than norepinephrine responses.

Proposed course of project: We have begun to investigate circulatory and catecholamine responses in patients with essential hypertension and in normotensives with a family history of hypertension, but too few have been studied so far to summarize the results. We will need actively to recruit participation of young patients with hypertension in whom removal of wisdom-teeth is clinically indicated, since only 2 hypertensives participated in this protocol in the past year. We have extended the study to normotensive children of hypertensive parents, since those children are at high risk for developing hypertension themselves. We wish to determine if those individuals show either pressor hyper-responsiveness or catecholamine hyper-responsiveness to non-physical stress. In collaboration with the NIDR, we also will assess the effects of sedation and adrenergic blockers on the circulatory and catecholamine responses.

Publications:

1. Goldstein, D.S.: Plasma norepinephrine during stress in essential hypertension. Hypertension 3:551-556, 1981.

2. Goldstein, D.S., Dionne, R., Sweet, J., Gracely, R., Brewer, H.B., Jr., Gregg, R. and Keiser, H.R.: Circulatory, plasma catecholamine, cortisol, lipid, and psychological responses to a real-life stress (third molar extractions): Effects of diazepam sedation and of inclusion of epinephrine with the local anesthetic. Psychosom. Med. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01930-03 HE

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Baroreflex Sensitivity in Hypertension

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	Bert Chidakel, B.S.	Engineer	BEIB R

COOPERATING UNITS (if any)

None

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Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.25	0.25	

CHECK APPROPRIATE BOX(ES)

XX (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are measuring baroreflex sensitivity in patients with essential hypertension and in normotensive controls using 8 different measurement techniques, in order to determine whether the techniques agree with each other, if baroreflex sensitivity is abnormal in essential hypertension, if clonidine exerts its antihypertensive action by affecting the baroreflex, if naloxone antagonizes clonidine's effects, and if normotensive children of hypertensive parents show any abnormalmality of baroreflex-mediated circulatory control.

PHS-6040
(Rev. 2-81)

Objectives: Since the baroreflex is the most powerful, rapidly acting circulatory homeostatic reflex, we have investigated whether an abnormality in the functioning of this reflex characterizes patients with essential hypertension. Because results of previous studies have disagreed about whether hypertensives show abnormal reflex sensitivity, and because any of several measurement techniques have been used, we compared eight techniques in the same subjects to determine the extent of agreement among them.

We also wished to test the hypotheses that the anti-hypertensive effects of clonidine are mediated by affecting baroreflex sensitivity, that naloxone antagonizes clonidine's anti-hypertensive effects by inhibiting endorphins, and that abnormalities of the baroreflex occur in normotensives likely to develop hypertension themselves in the future.

Methods: To conduct this study, a neck cuff apparatus was specially designed and fabricated to allow delivery of rapid changes in external neck pressures. The techniques used to assess baroreflex sensitivity are the change in R-R interval per unit change in systolic pressure during the Valsalva maneuver, upon release of the Valsalva maneuver, after injection of phenylephrine, after injection of nitroglycerine; the changes in R-R interval and in systolic pressure per mm Hg externally applied neck suction; and the changes in R-R interval and systolic pressure per mm Hg externally applied neck pressure. In some hypertensives, the baroreflex testing procedure is repeated after oral therapy with clonidine, and the effects of superimposed naloxone injection assessed.

Results and their significance: The average intercorrelation among these measures in 30 subjects was statistically significant ($r = 0.36$, $p < 0.01$), but suggests that, on average, variance in one measure accounted for only about 13% of the variance in others. Standard deviations across subjects often were as large as the means, indicating important inter-individual variability as well. Nevertheless, when age-matched subgroups of 10 hypertensives and 7 normotensives were compared, baroreflex rankings averaged across measurement techniques were significantly poorer in the hypertensives ($t = 3.27$ $p < 0.01$). Patients with essential hypertension therefore do appear to have diminished baroreflex sensitivity (1,2).

Three hypertensive patients have undergone repeat bororeflex testing after clonidine therapy. Clonidine decreased resting venous plasma norepinephrine by over 70% in these patients, resting systolic and diastolic pressures decreased to the normotensive range, and the overshoot in systolic pressure after release of the Valsalva maneuver was markedly attenuated, all these findings being consistent with effective therapeutic levels of clonidine. However, no obvious change in baroreflex sensitivity appeared. The data so far are inconsistent with the hypothesis that clonidine's anti-hypertensive effect derives from accentuated baroreflex sensitivity. No obvious effect of naloxone on blood pressure in clonidine-treated patients has appeared. An inverse relationship obtains between resting plasma norepinephrine and baroreflex-heart rate responsiveness. Since the former indicates sympathetic neural activity and the latter vagal responsiveness, those hypertensives with accentuated resting sympathetic tone may also show depressed vagal tone. These findings would be consistent with an abnormality of central nervous system control of blood pressure in some patients, resulting in a pattern of vagal inhibition and increased sympathetic outflow. The baroreflex protocol has been amended to allow re-testing of normotensive subjects after acute atropinization, in order to determine if pressor hyper-responsiveness can be produced in healthy people when vagal reflex or heart rate changes are blocked.

Proposed course of study: We wish to extend the study to allow statistical analysis of age-matched hypertensive and normotensive groups, to measure baroreflex sensitivity in patients with secondary hypertension, and to re-measure baroreflex function in treated hypertensives, to determine if the reflex abnormalities are part of the cause, or merely the result of essential hypertension. To date, only one patient with secondary hypertension has been tested, and only 2 patients with essential hypertension re-tested after anti-hypertensive therapy. Therefore, we will need actively to recruit these patients.

The observations with regard to clonidine and naloxone will be extended to an adequate number of patients to perform statistical analyses. We envision that these studies will take 1 to 2 years. We also will study baroreflex circulatory control in the normotensive children of hypertensive parents.

Publications:

1. Goldstein, D.S., Horwitz, D., and Keiser, H.R.: Comparison of techniques for measuring baroreflex sensitivity in man. Circulation (in press).

2. Goldstein, D.S., Horwitz, D., and Keiser, H.R.: Baroreflex sensitivity measured using several techniques in patients with essential hypertension and in normotensive controls. In: Jannetta, P.J., Dujovny, M., and Segal, R. (Eds.), Neurogenic Hypertension, Baltimore, Williams and Wilkins (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01932-02 HE

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Collaborative Studies of Plasma Catecholamines in Man

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	Elliot Gershen, M.D.	Chief, Section on Psychogenetics	BPB NIMH
	Ronald Polinsky, M.D.	Guest Worker	LCS NIMH
	Barbara Koch Eisenstein, M.D.	Rehabil. Medicine	Children's Hospital

COOPERATING UNITS (if any)

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LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Experimental Therapeutics

INSTITUTE AND LOCATION

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.15	0.15	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are assessing the effects of amphetamine and tyramine injections on blood pressure, heart rate, and plasma catecholamines in healthy volunteers, and relationships among plasma catecholamines, circulatory variables, and physical exertion in patients with hemophilia. These studies take advantage of the assay techniques developed and validated in this laboratory for measuring plasma catecholamines. We also have re-analyzed the raw data from another laboratory to assess possible age-dependence of hypertensive-normotensive differences in norepinephrine.

478

PHS-6040
(Rev. 2-81)

Objectives: D-amphetamine (d-A) and tyramine (Tyr) release norepinephrine from sympathetic neural storage vesicles. We wished to determine the extent of agreement between the pressure response--indicative of the increment in synaptic cleft norepinephrine after d-A or Tyr--and the circulating norepinephrine response--indicating "spillover" of norepinephrine from the cleft into the general circulation. Knowledge of these relationships may provide a basis for further study of pressor responses to endogenously released (as opposed to exogenously administered) norepinephrine in patients with hypertension.

Exercise training improves coagulation parameters in patients with hemophilia. It is possible that this benefit derives from the mediating effect of plasma epinephrine. We therefore are measuring plasma epinephrine in patients with hemophilia during exercise to determine the relationships, if any, with coagulation parameters.

Several recent literature analyses have concluded that hypertensive-normotensive differences in norepinephrine depend on the age group studied (1-3). When patients and controls averaged 40 years old or less, most of the studies reported significantly higher plasma norepinephrine in the hypertensives. We tested across a large number of individuals the hypothesis that the extent of hypertensive-normotensive differences in norepinephrine depends on patient age.

Methods: In the injection study, patients were infused with a single intraveous infusion of 0.3 mg/kg d-amphetamine over a 5-minute period or with graded bolus doses of tyramine to achieve an increment in systolic pressure of about 25 mm Hg. Blood pressure and heart rate were recorded during the subsequent 90 minutes and blood samples taken for assay of plasma catecholamines.

Venous plasma norepinephrine values from 191 resting, supine patients with a diagnosis of essential hypertension and from 129 normotensive controls were retrieved from WYLBUR files, in collaboration with Dr. C.R. Lake of the Uniformed Services University of the Health Sciences. The data were analyzed using the NIH SAS system to determine trends in norepinephrine with age in the hypertensive and normotensive groups.

In the hemophilia study, patients underwent bicycle ergometer exercise to pre-determined heart rate or systolic pressure criteria, and blood was drawn before, during, and after the exercise for determination of plasma catecholamines and coagulation parameters.

Results and their significance: Both d-A and Tyr dramatically increased systolic pressure (mean increases peaking at 39 and 36 mm Hg for d-A and Tyr), associated with increased plasma norepinephrine (NE) (224 and 149 pg/ml) but unassociated with changes in plasma epinephrine or heart rate (4). The time course of pressure and NE responses to Tyr was much shorter than to d-A, indicating that Tyr would be safer to use in testing hypertensive patients. The change in systolic pressure per unit change in NE was similar with the two drugs, consistent with the hypothesis that both agents increase pressure via increased synaptic cleft NE, and that circulating NE reflects "spillover" from the cleft into the general circulation.

Across 129 normotensives, plasma NE increased significantly with age, but did not across 191 hypertensives, due to relatively high NE values among young hypertensives. When patients and controls less than 40 years old were considered, hypertensives showed significantly higher NE than the controls (317 vs 245 ng/ml,

$t = 3.15$, $p < 0.01$), but above the age of 40, no significant hypertensive-normotensive difference was obtained. The data are consistent with increased sympathetic nervous system activity in the early stages of essential hypertension.

Eight healthy young adults and 10 patients with hemophilia have undergone the exercise testing procedure at Children's Hospital. Although the exercise produced predictable changes in epinephrine and norepinephrine concentrations, no correlated changes in coagulation paramenters have been noted. These results tend to refute the hypothesis that plasma epinephrine influences coagulation parameters in patients with hemophilia.

Proposed course of project: We have extended the d-amphetamine project to include catecholamine measurements in subjects pre-treated with adrenergic or dopaminergic blockers, in order better to understand the mechanism of pressor action of this sympathomimetic amine. We are planning to use the Tyr graded bolus procedure in a clinical protocol to determine whether patients with essential hypertension--or their normotensive children--show excessive pressor responses to endogenously released norepinephrine. We will continue to foster collaborative studies in which our contribution is the measurement of plasma catecholamines using liquid chromatography with electrochemical detection. The study of hemophiliacs is complete.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01933-02 HE

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Impedance Cardiography in Hypertension

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	Margaret Parker, M.D.	Sr. Staff Physician	MICU CC
	Raymond Dionne, DDS	Staff Fellow	NAB NIDR
	Peggy Wirdzek, RN	Nurse Specialist	NAB, NIDR

COOPERATING UNITS (if any)

Medical Intensive Care Unit - Clinical Center

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.10	0.10	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are determining the validity of impedance cardiography as a non-invasive measure of cardiac output and stroke volume in man, by comparison with results obtained using the Swan-Ganz thermodilution technique. If validated, the impedance cardiography technique offers the opportunity to measure cardiac output and peripheral resistance non-invasively in patients with essential hypertension.

481

PHS-6040
(Rev. 2-81)

Objectives: Impedance cardiography offers the unique advantage of determining cardiac output and stroke volume non-invasively on a beat-to-beat basis, potentially in ambulatory patients. The technique has not been validated to our satisfaction, though the equipment is commercially available. We therefore are testing the technique by comparison with results obtained using the Swan-Ganz thermodilution technique in the same patients.

Methods: Patients in the medical intensive care unit, or pre-operative patients, in whom invasive hemodynamic monitoring with a Swan-Ganz catheter is clinically indicated, undergo simultaneous measurement of cardiac output and stroke volume using impedance cardiography and Swan-Ganz thermodilution techniques.

Results and their significance: Across the 6 patients tested so far, cardiac outputs determined by the two techniques have agreed remarkably well--the correlation coefficient being more than 0.9. In one patient, who was undergoing hyperthermia as part of a cancer therapeutic protocol, excellent agreement was obtained across several measurements using impedance cardiography and the Fick technique ($r = 0.89$). These preliminary data agree with the results reported by others that impedance cardiography does validly measure cardiac output.

Proposed course of protocol: We will evaluate 10 patients using the two techniques. If the agreement across subjects continues to be excellent, then we will introduce manipulations designed to change peripheral resistance or cardiac output to determine the agreement between the techniques within subjects. If validated, the technique will be extremely important in studying cardiac output and peripheral resistance in patients with hypertension, since most investigators have reported increased peripheral resistance in essential hypertension and yet currently no available technique can measure non-invasively this crucial dependent variable. This study has been delayed partly because the NHLBI does not own the impedance cardiograph, which is used for NIDR clinical protocols. We strongly recommend that the NHLBI purchase the cardiographic apparatus, since it will play a pivotal role in clinical hypertension research in the near future.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01934-02 HE

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

The Sympathetic Nervous System and Hypertension

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	Irwin J. Kopin, M.D.	Chief	LCS NIMH
	Ronald Polinsky, M.D.	Guest Worker	LCS NIMH
	Alan Harris, Ph.D.	Div. Behavior. Biol.	Johns Hopkins Sch. of Med.
	Jaylan Turkkan, Ph.D.	Div. Behavior. Biol.	Johns Hopkins Sch. of Med.

COOPERATING UNITS (if any)

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LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Experimental Therapeutics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.30	0.30	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Using assay techniques developed and validated in this laboratory, we are measuring plasma norepinephrine as an indicator of sympathetic neural activity and plasma epinephrine as an indicator of sympathoadrenomedullary activity in hypertension.

483

PHS-6040
(Rev. 2-81)

Objectives: The results of recent literature reviews (1-4) suggest that venous plasma norepinephrine may be abnormally high in a proportion of patients with essential hypertension, suggesting in turn a pathogenetic role for accentuated sympathetic neural or sympathoadrenomedullary activity. We have developed and validated assay techniques for measuring plasma catecholamines (5-9), and we can now use these techniques for testing hypotheses about the role of the sympathetic nervous system in hypertension.

As part of the diagnostic testing in newly referred hypertensives, especially where pheochromocytoma is a possible diagnosis, we are conducting clonidine suppression testing to determine the contribution of sympathetic neural activity in causing their hypertension.

By measuring arm arterial and venous catecholamines, we can assess the contributions of peripheral vascular sympathetic activity as reflected by arteriovenous differences in norepinephrine, as well as presumed uptake of epinephrine by peripheral vessels as reflected by arteriovenous differences in epinephrine.

Because the concentration of norepinephrine in the synaptic cleft is the complex product of neuronal reuptake, metabolic degradation, nonspecific uptake, and release from pre-synaptic storage granules, knowledge about these specific components is crucial for understanding sympathetic nervous system function in essential hypertension. Using L- and D-norepinephrine, only the former of which is taken up into storage granules and recognized by MAO, stereoselective components of norepinephrine disappearance can be quantitated. Similarly, since both D- and L-norepinephrine are taken up by nerve terminals, while isoproterenol is not, the difference in disappearance kinetics between norepinephrine and isoproterenol should provide a measure of neural norepinephrine uptake.

Instrumental cardiovascular conditioning can produce large magnitude elevations in blood pressure in baboons. This preparation provides a unique model for study of chronically repeated pressor episodes where the extent of pressure elevation is controllable. We are determining the role of sympathetic activity in mediating the blood pressure changes.

Adie's syndrome is a neurologic abnormality of unknown cause, the signs of which are characteristic pupillary abnormalities, depressed deep tendon reflexes, and often orthostatic hypotension. We had the opportunity to study mechanisms of neural circulatory control in a patient with this syndrome, who also suffered severe hypertension of unknown cause.

Pheochromocytomas are catecholamine-secreting tumors which can cause hypertension. They represent the unusual situation of surgically curable hypertension. The diagnosis of pheochromocytoma has depended on urinary catecholamine metabolite measurement, arteriography, computed tomography, and exploratory laparotomy. We are determining the diagnostic usefulness of measuring blood levels of the direct products of pheochromocytoma secretion: plasma catecholamines.

Methods: In the study of arteriovenous differences in catecholamine, healthy persons and patients with essential hypertension undergo percutaneous insertion of plastic catheters into an antecubital vein and brachial artery. After 20 minutes lying supine, they undergo 10 cc phlebotomies, and the blood is collected into chilled, heparinized, evacuated tubes. Arterial and venous plasma catecholamine

concentrations are determined using liquid chromatography with electrochemical detection. For clonidine suppression testing, vital signs and plasma catecholamines are measured 3 hours after a single oral dose of 0.3 mg clonidine.

In the study of norepinephrine kinetics, healthy persons and patients with essential hypertension undergo insertion of catheters or needles into the antecubital vein in each arm. C-14 labelled D-norepinephrine, tritiated L-norepinephrine, and tritiated isoproterenol are infused together for 20 minutes, and blood samples taken during the infusion and for up to 60 minutes afterwards. The norepinephrine and isoproterenol peaks are separated using liquid chromatography with electrochemical detection and the C-14 and tritium counted using liquid scintillation spectrometry. The ratio of C-14 to tritium in norepinephrine provides a measure of the stereoselective components of norepinephrine disappearance, while the ratio of tritium in isoproterenol to that in norepinephrine provides a measure of neuronal norepinephrine uptake.

In the study of plasma catecholamines during instrumental cardiovascular conditioning, baboons undergo conditioning with reinforcement for increases in diastolic blood pressure during daily, 12-hour training sessions. The criterion blood pressure is increased over the course of several weeks using a shaping procedure. Plasma catecholamines are measured using liquid chromatography with electrochemical detection on blood samples drawn from chronically indwelling arterial catheters before and during the conditioning sessions.

In the study of neural circulatory control in Adie's syndrome, we measured plasma catecholamines during supine rest, during standing, and after insulin-induced hypoglycemia; arteriovenous differences in plasma catecholamines; plasma disappearance kinetics of injected radiolabelled D- and L-norepinephrine; and measured baroreflex sensitivity using 8 different techniques.

Results and their significance: So far, we have measured arteriovenous catecholamine differences in 14 normotensives and 15 patients with essential hypertension. Hypertensives have shown higher arterial and venous norepinephrine levels (268 vs. 158 pg/ml and 294 vs. 172 pg/ml) than normotensives, as well as greater arteriovenous differences in norepinephrine (26 vs. 15 pg/ml). Hypertensives have also shown higher arterial and venous epinephrine levels (106 vs 55 pg/ml and 59 vs. 38 pg/ml), and an accentuation of the arteriovenous difference in epinephrine (37 vs. 17 pg/ml).

These results suggest that accentuated peripheral vascular sympathetic activity occurs in essential hypertension, and that both abnormal sympathetic neural and sympathoadrenomedullary activity characterize a proportion of patients with essential hypertension. Too few patients have undergone clonidine suppression testing to summarize the results here.

In the study of norepinephrine kinetics, 10 hypertensive and 12 normotensive subjects have been studied. Although supine, resting, pre-infusion venous plasma norepinephrine levels were significantly higher in the hypertensives, the hypertensive and normotensive groups did not differ significantly in the disappearance kinetics of L- or D-norepinephrine or isoproterenol. The overall increased resting plasma norepinephrine levels seen in essential hypertension therefore appear to derive from increased sympathetic neural activity, not diminished disappearance of plasma norepinephrine. Because isoproterenol is not taken up by nerve endings, the ratio of ^{3}H-L-norepinephrine increased after the infusion ended. This

increase was almost completely abolished by pre-treatment with the neuronal norepinephrine uptake blocker, desipramine, in 5 subjects. Thus, changes in the isoproterenol:norepinephrine ratio after simultaneous infusion of both provide an index of neuronal norepinephrine uptake in man.

In the study of instrumental diastolic blood pressure conditioning in baboons (10), pre-training baseline norepinephrine averaged 364 pg/ml, epinephrine 253 pg/ml, dopamine 270 pg/ml, and renin activity 2.6 mg/ml/hr. During the training sessions, systolic pressure increased significantly 19 mm Hg (15%), diastolic pressure 20 mm Hg (30%), and heart rate 33 bpm (33%) compared with pre-session values. The blood pressure elevations were completely reversible, demonstrating that chronically repeated pressor episodes in otherwise healthy individuals do not themselves cause sustained hypertension. During the session, norepinephrine increased significantly by 253 pg/ml (48%), and epinephrine 130 pg/ml, while dopamine and renin activity were unchanged. Plasma norepinephrine correlated positively with systolic and diastolic pressures in all baboons tested, in both baseline and training conditions. To the extent that plasma norepinephrine reflects sympathetic neural activity, these findings demonstrate an association between sympathetic activity and blood pressure in this hypertensive model.

In the study of the patient with Adie's syndrome, resting norepinephrine levels were normal, and the patient showed a normal increment in norepinephrine with standing. Baroreflex sensitivity was markedly depressed, by all techniques in which R-R interval was a dependent measure, yet heart rate responses to isoproterenol and atropine were essential normal. Data derived from the norepinephrine infusion have not been analyzed yet. The results suggest an abnormality of central integration of reflex circulatory control in this syndrome.

Across 15 patients with surgery-proven pheochromocytoma and 41 with hypertension but without pheochromocytoma, the plasma norepinephrine distributions showed only slight overlap. Two patients with pheochromocytoma showed plasma norepinephrine levels within two standard deviations of the mean of the non-pheochromocytoma patients; the remainder showed plasma norepinephrine levels exceeding 2 SD's above the non-pheochromocytoma mean. These results suggest that exceedingly high norepinephrine levels--above 600 pg/ml--in resting, supine patients with hypertension support the diagnosis of pheochromocytoma, but that levels less than about 600 pg/ml do not exclude the diagnosis.

Proposed course of project: The study of arteriovenous catecholamine differences will be continued until about 20 normotensives and 20 hypertensives have been included. The study of norepinephrine kinetics and of clonidine suppression will be continued until about 15 patients with hypertension have been tested. These studies should be completed in 1 to 2 years. We will, in addition, test normotensive children of hypertensive parents under these protocols.

Because the conditioning procedure in baboons has not produced sustained hypertension when the sessions have ended, we wish to determine whether salt loading interacts with the conditioning to produce sustained hypertension. We also will repeat catecholamine measurements in baboons trained to decrease diastolic pressure. These studies should take 2 to 3 years.

Plasma catecholamine determinations in patients in whom the diagnosis of pheochromocytoma is entertained will be conducted as an ongoing clinical service.

In general, we anticipate that our studies of plasma catecholamines will be expanded to other cardiovascular diseases, since the sympathetic nervous system has been invoked to explain a wide variety of pathophysiologic phenomena in clinical cardiology (9). We hope to conduct such studies in collaboration with the Cardiology Branch in the future. Finally, in collaboration with Drs Kopin and Polinsky at NIMH, we plan to estimate synaptic cleft norepinephrine concentrations in man, using norepinephrine and tyramine infusions and orthostatic stress.

Publications:

1. Kopin I.J., Goldstein, D.S., Feuerstein, G.Z.: The sympathetic nervous system and hypertension. In: Laragh, J.J., Buhler, F.R., and Seldin, D.W. (Eds.), Frontiers in Hypertension Research. NY: Springer-Verlag, 1981.

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6. Goldstein, D.S., Feuerstein, G.Z.: Improved reliability of the liquid chromatography-electrochemical detection assay technique for measuring plasma epinephrine. Clin. Chem. 27:508, 1981.

7. Shoup, R.E., Kissinger, P.T., Goldstein, D.S.: Rapid liquid chromatographic methods for assay of norepinephrine, epinephrine, and dopamine in biological fluids and tissues. In: Ziegler, M.G., and Lake, C.R. (Eds.), Norepinephrine: Clinical Aspects, Baltimore: Williams and Wilkins, 1982 (in press).

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9. Lake, C.R., Ziegler, M.G., Goldstein, D.S., and Chernow, B.: Indices of sympathetic nervous system function in man. In: Ziegler, M.G., and Lake, C.R., (Eds.), Norepinephrine: Clinical Aspects, Baltimore: Williams and Wilkins, 1982 (in press).

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11. Goldstein, D.S.: Plasma norepinephrine as an indicator of sympathetic neural activity in clinical cardiology. American Journal of Cardiology, 48:1147-1154, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01937-02 HE

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Modification of Endotoxic Shock by Vagotomy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Arthur B. Pitterman, M.D.	Medical Staff Fellow	HE NHLBI
	Daniel J. Goldstein, M.D.	Expert	HE NHLBI
OTHERS:	Harry R. Keiser, M.D.	Deputy Chief	HE NHLBI
	Gerald Kelly	Biol Lab. Tech.	HE NHLBI
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COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension - Endocrine Branch

SECTION
Experimental Therapeutics

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	.75	.25

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Bilateral subdiaphragmatic vagotomy does not improve the clinical course nor the survival of Sprague-Dawley rats injected intravenously with E. coli lipopoly-saccharide. These results show that whatever peripheral signals are elicited by endotoxin to generate the centrally mediated hypotensive response, they are not conveyed to the central nervous system by abdominal afferent fibers.

PHS-6040
(Rev. 2-81)

Objectives: The mechanism by which the gut and brain peptides have central effects after systemic injection remain to be understood (Life Sci. 25 401-414, 1979). At least in two instances, angiotensin II induced drinking (Proceedings of the VII International Congress of Pharmacology, Pergamon Press, London, 1981) and cholecystokin induced satiety (Science 213 1036-37, 1981). Afferent abdominal vagal fibers seem to be involved in the transmission of peripherally originated signals to the central nervous system. It is known that enkephalins and β-endorphins exert central effects after systemic administration, which last long after the peptides have disappeared from the blood stream (Life Sci. 25 401-414, 1979). The hypotension induced by morphine in the anesthesized rat may be blocked by cervical vagotomy (Brit. J. Pharmacol. 7 542-552, 1952). The dramatic reversal by naloxone of cardiovascular consequences of hypovolemic, endotoxic, and spinal shock models have awakened interest in the role of endogenous opioids in the regulation of blood pressure. It is not known if circulating opioid peptides are involved in the shock process. Our objective is to study the effects of bilateral subdiaphragmatic vagotomy on the hypotensive effects of endotoxin in the rat, as well as the consequences of the interruption of the abdominal vagal pathway on the survival after endotoxic shock.

Methods: Endotoxic BP Response in Vagotomized Rats. Male Sprague-Dawley rats 275-350g underwent either abdominal vagotomy (bilaterally) or sham operation. Three weeks post surgery indwelling venous and arterial catheters (PE 50) were inserted into the left jugular vein and tail artery. The rats were placed in individual cages in which they were free to move about with the catheters exiting from the back of their neck. Twenty four hours after implantation of the catheters, the arterial catheter was connected to a pressure transducer and recorder. Lipopolysaccharide 70 mg/kg was administered via the venous catheter. The blood pressure was recorded for 2 hours after the lipopolysaccharide injection and the animals were observed 24 hours later for survival.

Endotoxic Mortality in Unrestrained Rats. Three to six weeks after vagotomy and sham operations, a jugular catheter was inserted as previously described. Each animal was placed in a cage and 24 hours later the rat received endotoxin 100 mg/kg via the catheter. The number alive at 24 hours was determined.

Endotoxic Mortality in Restrained Rats. Three to six weeks after vagotomy and sham operation the rats were placed into a restraining cage for approximately one minute while administering endotoxin 70 mg/kg IV. The number alive at 24 hours was determined.

Endotoxic Mortality in Atropine Methylnitrate Treated Rats. Sprague-Dawley male rats 300-350g were anesthetized (Ether USP) prior to insertion of a (PE 50) catheter into the jugular vein. Twenty four hours later the rats received either atropine methyl nitrate 1 mg/kg IV, or saline followed by endotoxin 70 mg/kg IV. The cannula was then connected to an infusion of atropine methynitrate, 1 mg/kg/h or saline 0.0035 ml/min. The infusion was continued for 24 hours and then the number alive was counted.

Results: There was no significant differences between vagotomized and sham operated rats in systolic, diastolic, or mean arterial blood pressure during the endotoxin induced hypotension. Mortality was not significantly changed by vagotomy. Atropine methylnitrate did not protect after endotoxin.

Significance of findings: These results show that whatever peripheral signals are elicited by endotoxin that are involved in the central mediated hypotensive response, they are not conveyed to the central nervous system by vagal abdominal afferent fibers.

Proposed course of study: We will compare the effect of I.V. thyrotropin releasing hormone (TRH) on endotoxic shock, in sham operated and vagotomized animals, to evaluate the role of the abdominal vagal afferent fibers in the transduction of the signals elicited by the peripherally administered peptide.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01944-01 HE

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Effects on Blood Pressure of Arginine Vasopressin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Eric S. Marks, M.D.	Guest Worker	HE NHLBI
OTHER:	Harry R. Keiser, M.D.	Deputy Chief, Hypertension-Endocrine Branch	HE NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

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NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.6	.6	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are evaluating the mechanism of the blood pressure response induced by osmotic and nonosmotic stimuli of arginine vasopressin (AVP). All experiments are carried out in awake, unrestrained, bilaterally nephrectomized rats. Hypertonic saline administered to the Long Evans strain induced a significant increase in mean blood pressure which was not observed in the Brattleboro strain of rat. A dependence on the presence of arginine vasopressin appears to exist. Naloxone, an opiate antagonist, had no effect on this rise in the Long Evans rats.

PHS-6040
(Rev. 2-81)

OBJECTIVES:

We seek to define the role of AVP in the control and maintenance of blood pressure during physiologic manipulations which affect both vascular volume and plasma osmolarity. The rise in blood pressure noted with the administration of hypertonic saline (Am. J. Physiol. 240:H287, 1981) is partially reversed following the administration of a synthetic blocker of AVP. We have chosen to use the Brattleboro strain which has a complete congenital absence of AVP and its intact parent strain, Long Evans, to avoid the problems of incomplete inhibition and partial interaction. Our experimental design will provide the means for defining AVP dependence.

METHODS:

Adult rats are unilaterally nephrectomized one week prior to use. On the day preceding the study catheters are placed in the tail artery and the external jugular vein and the remaining kidney is removed. Rats receive either hypertonic saline or Ficoll 70. The former acts as an osmotic stimulus to AVP release while the latter, by reducing intravascular volume, is a non-osmotic stimulus. Hypertonic saline (1.5 mEq/ml) is infused over two hours to give a total volume of 2 mls. Ficoll 70 (6%) is administered through an indwelling peritoneal catheter. Naloxone was given as a 2 mg/kg loading dose followed by a 20 µg/kg/min infusion incorporated in the hypertonic saline solution. Blood pressure is monitored continuously and blood samples are obtained at the end of each infusion to measure AVP, norepinephrine, sodium and osmolarity. Dose response curves to 20, 40, 100 and 200 ng bolus doses of norepinephrine were generated before and after the hypertonic infusion.

RESULTS:

The administration of hypertonic saline increased mean blood pressure in the Long Evans group by 31±15 mm Hg. Treatment with Naloxone had no significant effect with an increase of 31.00±13 mm Hg. In Brattleboro rats blood pressure increased by 5±3 mm Hg, significantly less than that in the intact strain. Preliminary dose response curves reveal an apparent increase in sensitivity to infused norepinephrine following hypertonic saline infusion.

PROPOSED COURSE OF STUDY:

We intend to extend the number of animals in each group. The AVP dependence of blood pressure in the peritoneal implantation group will be studied. The effect of the AV3V cerebral lesion will be evaluated in this system to further define its inhibitory effect on blood pressure and relationship with AVP. The vasoconstrictive action of AVP may be of physiologic importance under conditions of acute hemodynamic stress, such as decompensated congestive heart failure and in patients who undergo extracorporeal circulation.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01945-01 HE

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Catecholamine Production by the Kidney

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Eric S. Marks, M.D.	Guest Worker	HE	NHLBI
	David Goldstein, M.D.,Ph.D.	Clinical Associate	HE	NHLBI
OTHER:	Harry Keiser, M.D.	Deputy Chief	HE	NHLBI
	Gerald Kelly	Biol. Lab. Tech.	HE	NHLBI

COOPERATING UNITS (if any)

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Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

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NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.4	.3	.1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The production and release of catecholamines by the kidney are being studied in the anesthetized dog. We are using simultaneous sampling from the aorta, renal veins, and ureters. This is done in conjunction with measurements of arterial blood pressure, renal blood flow and glomerular filtration rate to allow for an overall assessment of these physiologic interactions. The effect of an acute reduction in renal blood flow to one kidney on the above parameters is also being evaluated.

PHS-6040
(Rev. 2-81)

Objectives: The role of the kidney in the production of catecholamines and their subsequent effect on renal and systemic hemodynamics is not well defined. Production of norepinephrine by the kidney has been documented by other workers (Am J Physiol 239:F371, 1980). The intent of our studies is to: 1) simultaneously measure the production and excretion of these catecholamines by the kidney, and 2) correlate their levels in plasma and urine with glomerular filtration rate, renal blood flow, and excretion of solute and water. In addition, we will study the effect of unilaterial renal artery obstruction on catecholamine production by the kidney and its effect on systemic blood pressure.

Methods: Female dogs (20-24 kg) are anesthetized with sodium pentobarbital and placed on controlled ventilation. Catheters are placed for arterial, venous, and ureteral sampling. An electromagnetic flow probe is placed on the left renal artery. A constant infusion of Lactated Ringers solution at 4.5 ml/min is begun and both inulin and PAH are infused at the appropriate rate. All blood losses are replaced with blood from donor dogs. A constricting snare is placed around the left renal artery, with care not to crush the major renal nerves. This provides a reduction in blood flow of 40-45%. Unilateral renal denervation is performed by careful dissection and sectioning of the nerve followed by application of phenol in absolute alcohol.

Results: Difficulties with the stability and viability of the animal preparation have been overcome. Preliminary results confirm the kidney as a source of norepinephrine production. The AV difference for epinephrine shows a moderate decrease across the kidney, with variable changes noted for dopamine. At present there is insufficient data to correlate plasma and urine levels, or to comment on their relationship with observed changes in renal function. It is of note, however, that the induced acute reduction in renal blood flow was associated with a reduction in renal output of norepinephrine and an increase in dopamine from the affected side.

Proposed course of the study: Our preliminary results demonstrate the utility of this preparation. We intend to increase the number of experimental animals to confirm our initial findings. In addition, we plan to study chronic Goldblatt 2 kidney one clip dogs to follow the course and effect of this lesion on renal catecholamine activity.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01946-01 HE

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Deficient platelet α-adrenoceptor function in patients with Bartter's syndrome.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	John R. Gill, Jr., M.D.	Senior Investigator	HE NHLBI

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Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics Section

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National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In patients with Bartter's syndrome (hypokalemic alkalosis, hyperreninemia, aldosteronism, increased adrenergic nervous system function, hyperprostaglandinuria, normal blood pressure) cyclic AMP content in platelet-rich plasma is supranormal and platelet aggregation is defective. It has been proposed that a stable prostaglandin (PG) related to prostacyclin, which is overproduced in Bartter's syndrome and is a potent antiaggregatory substance, is responsible for stimulation of cyclic AMP formation and impaired platelet aggregation. Evaluation of the production of cyclic AMP by platelets in Bartter's syndrome and in normal subjects showed that in vitro basal, PGE_1-stimulated, and PGE_1-stimulated, norepinephrine-inhibited cyclic AMP production by platelets in Bartter's syndrome was subnormal. The affinity of and binding by platelet alpha (α) receptors was normal. Treatment with indomethacin, an inhibitor of prostaglandin synthesis corrected only basal cyclic AMP production. These results indicate that in Bartter's syndrome cyclic AMP production by platelets is impaired and suggest that platelets are not the source of the increased plasma cyclic AMP concentration that probably decreases platelet aggregation in this disorder.

496

PHS-6040
(Rev. 2-81)

Project Description and Objectives:

A consistent finding in patients with Bartter's syndrome is a decreased responsiveness of platelets to aggregating agents such as ADP, epinephrine, thrombin, and collagen and this subnormal aggregation is thought to be the result of supranormal plasma cyclic AMP. This subnormal platelet aggregation appears to be secondary to a circulating factor since it can be induced in normal platelets by suspending them in plasma from a patient with Bartter's syndrome. Treatment of patients with Bartter's syndrome with inhibitors of prostaglandin synthesis, such as indomethacin, corrects the abnormal platelet aggregation and indicates that a prostaglandin may mediate it. The defect can also be corrected in vitro by antibodies against prostacyclin suggesting that prostacyclin may be the culprit. Recently, it has been reported that plasma 6-keto-prostaglandin E_1, a stable, biologically active metabolite of prostacyclin is normal in patients with Bartter's syndrome and it was suggested that other prostaglandins that inhibit platelet aggregation such as prostaglandin E_1 or prostaglandin D_2 rather than prostacyclin may cause the defect in platelet aggregation in patients with Bartter's syndrome.

In order to elucidate the mechanism leading to the increase in plasma cyclic AMP in Bartter's syndrome we studied the regulation of cyclic AMP production by platelets in Bartter's syndrome. Since Bartter's syndrome is associated with an increase of adrenergic activity and since α-adrenergic agonists inhibit platelet cyclic AMP production, we also measured platelet α-receptors in these patients.

Methods Employed:

Six women with Bartter's syndrome and seven normal women were studied. The patients were admitted to an air-conditioned ward at the Clinical Center of NIH and were fed a constant metabolic diet containing 109 mEq/day sodium. All medications had been discontinued at least 2 weeks before hospital admission. All subjects were studied during a control period and after treatment with indomethacin (INDO) (150 mg/day for 5 days) to inhibit prostaglandin production.

Platelets were prepared from fresh blood samples. Membranes for measurement of binding of [^{3}H] dihydroergocryptine (DHE), an index of the number of α-receptors, were prepared by centrifugation of platelet-rich plasma (PRP) at 12,000 g for 10 min. Cyclic AMP production in platelets under basal conditions and in response to a known stimulant (PGE_1) as well as in response to an inhibitor (norepinephrine) was measured by a protein-binding assay. In two patients the affinities of platelet α-receptors were measured in saturation experiments and calculated by scatchard analysis.

Major Findings and Significance:

The control observations and results after treatment with indomethacin both in patients with Bartter's syndrome and in normal subjects are summarized in the the following table:

	Dihydroergocryptine binding (fmol/mg protein)	cAMP production (pmol/mg protein/min) Basal	PGE_1	PGE_1+NE
Normal	129±7 mean ± SE	5.1±0.9	33.3±4.1	14.4±2.0
Normal + INDO	183±22	2.8±0.9	36.0±3.0	15.4±2.7
B.S.	180±56	2.2±0.6*	6.2±2.5+	4.0±1.1**
B.S. + INDO	315±85	7.0±2.3	11.8±3.3**	8.3±1.5*

*p<0.05; **p<0.01; +p<0.001 compared to normal women

Scatchard analysis showed that the affinity of platelet α-receptors in Bartter's syndrome was not different from that in normal controls. Also, binding by platelet α-receptors in patients with Bartter's syndrome was normal. In contrast, cyclic AMP production in Bartter's syndrome was significantly subnormal; PGE_1-stimulated and PGE_1-stimulated, norepinephrine-inhibited cyclic AMP production by platelets were 20% and 30%, respectively, that of normal controls. Inhibition of prostaglandin synthesis by treatment of the patients with indomethacin did not correct the defect in platelet function.

Two major conclusions can be drawn from the present data. First, platelets can be excluded as the source of the increase in plasma cyclic AMP concentration in patients with Bartter's syndrome. Second, the increase in adrenergic activity in Bartter's syndrome does not lead to a "downregulation" of α_2-receptors.

The available evidence indicates that platelets tend to aggregate when cyclic AMP production is decreased. The observation that aggregation is defective in Bartter's syndrome, therefore, suggests that the cause of the defect is, indeed, a "circulating" factor. It is possible that the increase in plasma cyclic AMP in the syndrome is mediated by increased cyclic AMP release from endothelial cells of blood vessels. Prostacyclin is the principal prostaglandin synthesized in vascular endothelial cells. It is, therefore, likely that prostacyclin, which is overproduced in Bartter's syndrome, stimulates cyclic AMP production in the endothelium, thus causing the defect in platelet aggregation.

Measurement of plasma 6-keto-PGE_1 may not be a reliable index of prostacyclin production since prostacyclin is rapidly excreted and metabolised into other metabolites and since only small amounts of 6-keto-PGE_1 may reach the circulation. Increased prostacyclin production does appear to be reflected by urinary 6-keto-PGE_1.

Publications:

1. Dunnick, N.R., Doppman, J.L., Gill, J.R., Jr.: Failure to ablate the adrenal gland by injection of contrast material. Radiology 142: 67-69, 1982.

2. Dunnick, N.R., Doppman, J.L., Gill, J.R., Jr., Strott, C.A., Keiser, H.R. Brennan, M.F.: Localization of functional adrenal tumors by computed tomography and venous sampling. Radiology 142: 429-433, 1982.

3. O'Leary, T.J., Liotta, L.A., Gill, J.R., Jr: Pigmented adrenal nodules in Cushing's syndrome. Arch. Pathol. Lab. Med. 106: 257, 1982.

4. Gullner, H.-G., Gill, J.R., Jr., Mitchell, M.D.: Plasma 6-keto-prostaglandin $F_{1\alpha}$ in disorders involving hypokalemia. Prostaglandins, Leukotrienes and Medicine, in press.

5. Gill, J.R., Jr.: The role of chloride transport in the thick ascending limb in the pathogenesis of Bartter's syndrome. Klinische Wochenschrift, in press.

6. Gill, J.R., Jr.: Pathogenesis of Bartter's syndrome: Controversial aspects and present status. Controversies in Nephrology 1981, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01947-01 HE

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Role of Prostaglandins in the Natriuretic Response to Acetylcholine in Indomethacin-treated Dogs.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	John Yun, Ph.D.	Guest Worker	HE NHLBI
OTHER:	John R. Gill, Jr., M.D.	Senior Investigator	HE NHLBI
	Harry R. Keiser, M.D.	Deputy Chief	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Experimental Therapeutics Section

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	1.5	1.5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The effect of prostaglandin E_2 and $F_{2\alpha}$ on renal function was examined in indomethacin-treated dogs receiving an intrarenal infusion of acetylcholine.

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Project Description and Objectives:

Infusion of acetylcholine (ACh) into the renal artery of the dog is known to increase sodium and water excretion. How ACh produces diuresis and natriuresis is not clear. Recently, we observed that a requirement for maintenance of the diuretic and natriuretic response to ACh is an intact synthesis of prostaglandins. In that study, renal arterial infusion of ACh in control dogs produced a sustained diuresis and natriuresis, whereas in the dogs pretreated with indomethacin it produced only a transient diuresis and natriuresis. If the diuretic and natriuretic response to ACh depends on prostaglandins, then in dogs pretreated with indomethacin, infusion of prostaglandins should restore the response to ACh to normal. The present study examines the effect of renal arterial infusion of prostaglandin E_2 and $F_{2\alpha}$ (PGE_2 and $PGF_{2\alpha}$) on the diuretic and naturetic response to ACh of indomethacin-treated dogs.

Methods Employed:

Mongrel dogs were given a diet containing 180 mEq/day of sodium for one week before study. Renal clearances, with periods of 20 minutes, were performed according to the following protocols:

Group I: Intrarenal infusion of ACh:

After three consecutive 20-minute control clearance periods were obtained, ACh was infused into the left renal artery at 40 µg/min for five 20-minute periods. Left renal arterial infusion of saline was then resumed for two 20-minute post-control periods.

Group II: Intravenous injection of indomethacin plus intrarenal infusion of ACh:

After a 20-minute control clearance period was obtained, a bolus of indomethacin (5 mg/kg) was injected into a femoral vein and two 20-minute clearance periods were obtained. ACh was then infused into the left renal artery at 40 µg/min for five 20-minute periods. Saline was then infused into the left renal artery for two 20-minute post-control periods.

Group III: Intravenous injection of indomethacin plus intrarenal infusion of PGE_2 and ACh:

After a 20-minute control period was obtained, a bolus of indomethacin (5 mg/kg) was injected into a femoral vein, and simultaneously, PGE_2 was infused into the left renal artery at 1.9 µg/min. Two 20-minute clearance periods were collected. ACh (40 µg/min) was then infused into the left renal artery for five 20-minute periods. Infusion of ACh was then stopped while the intrarenal infusion of PGE_2 was continued for two 20-minute post-control periods.

Group IV: Intravenous injection of indomethacin plus intrarenal infusion of $PGF_{2\alpha}$ and ACh:

The protocol in this series of experiments is the same as that in group III except that $PGF_{2\alpha}$ (1.9 µg/min), instead of PGE_2, was infused into the left renal artery.

Major Findings and Significance:

Renal arterial infusion of acetylcholine (ACh) (40 μg/min) produces a natriuresis and diuresis and an increase in renal plasma flow (RFP) without a change in glomerular filtration rate (GFR) or renin secretion rate (RSR). To determine the role of prostaglandins in this natriuretic response to ACh, the effect of an inhibitor of prostaglandin synthesis was examined. In dogs pretreated with indomethacin (Indo) 5 mg/kg, i.v.) renal arterial infusion of ACh produced an increase then a decline in urinary flow and sodium excretion that was accompanied by a progressive fall in GFR and RPF, and a progressive increase in RSR. Renal arterial infusion of PGE_2 (1.9 μg/min) but not $PGF_{2\alpha}$ (1.9 μg/min) before and during the infusion of ACh restored the diuretic and natriuretic response to ACh in Indo-treated dogs. The data suggest that the normal diuretic and natriuretic response to ACh requires normal synthesis of prostaglandins, probably PGE_2 but not $PGF_{2\alpha}$.

Publications:

1. Yun, J.C.H., Gill, J.R., Jr., Bartter, F.C., Kelly, G.D., Keiser, H.: Effect of bradykinin on renal function in dogs treated with indomethacin or propranolol. Renal Physiol. 5: 31-43, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01948-01 HE

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Pro-opiolipomelanocortin Derived Peptides in the Regulation of Steroid Production.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Hans-Georg Gullner, M.D.	Guest Worker	HE NHLBI
Other:	John R. Gill, Jr., M.D.	Senior Investigator	HE NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics Section

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

There is considerable indirect evidence that a pituitary factor other than ACTH participates in the regulation of steroid production in the adrenal cortex. Recently, it has been shown that a large precursor peptide is present in the pituitary gland which contains the amino acid sequence for ACTH, the lipotropins and the endorphins (Proopiolipomelanocortin, POLMC). Beta-endorphin is the most potent endorphin with regard to its opioid activity. We examined, therefore, the effects of β-endorphin (β-LPH 61-91) and of several of its N- or C-terminal fragments on steroidogenesis in the in situ perfused adrenal gland of hypophysectomized dogs. We found that β-endorphin selectively stimulates the secretion of aldosterone without affecting that of cortisol, with a potency similar to that of ACTH (β-LPH 1-39). Met-enkephalin (β-LPH 61-65), α-endorphin (β-LPH 61-76) and γ-endorphin (β-LPH 61-77) had no effect on steroid production. However, β-EP-(β-LPH 43-91) was active in stimulating aldosterone secretion rate. This indicates that the active core of β-endorphin resides in the C-terminal portion of the molecule. The results suggest that β-endorphin may play a role in the regulation of adrenal steroidogenesis.

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Project Description and Objectives:

Steroid production is regulated by ACTH, angiotensin II and potassium. Considerable indirect evidence indicates that, in addition to ACTH, an unknown pituitary factor may regulate aldosterone secretion. It has been speculated that one of the POLMC-derived pituitary peptides could be that factor. Recently, β-lipotropin (β-LPH) and β-melanocyte-stimulating hormone (β-MSH) were observed to stimulate aldosterone production in collagenase-dispersed rat capsular adrenal cells without affecting corticosterone production in zona fasciculata cells. Beta-endorphin and met-enkephalin had no effect on steroid production in this system. Other investigators observed stimulation of both aldosterone and cortisol and have suggested that the effects observed with β-LPH were due to a contamination of the β-LPH preparation with ACTH and that the remainder of the steroidogenic activity was due to the heptapeptide core sequence common to ACTH, β-MSH and β-LPH and not due to a specific action of β-LPH. Beta-endorphin is the most potent of the pituitary peptides with regard to its pharmacological activity. We studied, therefore, the effects of infusion of β-endorphin and of β-END fragments into the in vivo perfused adrenal glands of hypophysectomized dogs on the production of aldosterone and cortisol and compared them with those of ACTH.

Methods Employed:

Female foxhounds were fed a constant diet containing 263 mEq/day of sodium. On the day before and on the morning of the experiment the animals received 1.0 mg dexamethasone sodium phosphate as an intramuscular injection. On the day of the experiment, the pituitary gland was removed and the adrenal glands prepared for perfusion by a technique which isolates the circulation of the glands on the arterial and venous sides. The adrenal glands were perfused via an arterial "pouch" by the animal's own heart without interruption of blood supply. Blood for hormone determinations was collected from a venous pouch that drained the adrenal veins.

The peptides (ACTH, β-END, α-END, γ-END and met-enkephalin) were infused in equimolar concentrations (3 pmoles/min) for a period of 50 minutes. Buffer or normal saline was infused during control and post-control periods. Plasma concentrations of aldosterone and cortisol were measured by specific and sensitive radioimmunoassays. Secretion rates of aldosterone and cortisol were calculated from adrenal blood flow rate and steroid concentrations. To exclude the possibility that changes in plasma potassium occurred which might affect aldosterone secretion, we also measured potassium concentrations in adrenal venous plasma.

Statistical analysis was done by one-way analysis of variance.

Major Findings and Significance:

The results of infusion of ACTH, β-endorphin and met-enkephalin are shown in the table below:

Effect on Aldosterone Secretion (ng/min)

	Control (30min)	Infusion (50min)	Post-control (60min)
ACTH	3.98 ± 1.12	12.56 ± 2.34*	9.98 ± 3.07
β-endorphin	2.43 ± 0.54	10.96 ± 3.69*	10.2 ± 5.4
met-enkephalin	4.51 ± 3.06	5.03 ± 3.50	5.04 ± 3.33

Effect on Cortisol Secretion (μg/min)

	Control (30min)	Infusion (50min)	Post-control (60min)
ACTH	0.13 ± 0.03	3.15 ± 0.69**	0.32 ± 0.08
β-endorphin	0.08 ± 0.02	0.09 ± 0.02	0.11 ± 0.02
met-enkephalin	0.59 ± 0.12	0.70 ± 0.16	0.73 ± 0.17

*P<0.05; **P<0.01

Potassium concentration in venous plasma did not change significantly with infusion of any peptide (3.6±0.2 mEq/L at the end of the control period, 3.4±0.3 mEq/L at the end of β-endorphin infusion).

Infusion of β-endorphin into the adrenal gland of hypophysectomized, nephrectomized dogs caused a significant increase in aldosterone secretion but not cortisol secretion. Infusion of met-enkephalin had virtually no effect on either aldosterone or cortisol secretion rates. Equimolar concentrations of (1-39) ACTH, which elicited equal percent increases in aldosterone and cortisol secretion, produced an absolute increase in aldosterone secretion similar to that of β-endorphin, indicating that the two peptides are equipotent. The smallest concentration of β-END that significantly increased aldosterone secretion was 0.3 pmol/min. Measurement of β-endorphin-like immunoreactivity showed concentrations in the range of about 350 pg/ml. Adrenal blood flow is about 8 ml/min. This suggests that an infusion of β-endorphin at a rate of 0.3 pmoles/min or 1 ng/min increased the concentration of the peptide in adrenal blood by about 30%. This would indicate that circulating concentrations of β-endorphin may be high enough to stimulate aldosterone secretion. Therefore, β-endorphin may play a physiological role in the regulation of aldosterone secretion.

Proposed Course of Project:

Experiments are presently in progress to examine the effects of smaller fragments of β-endorphin on aldosterone secretion rates in an attempt to define the amino acid sequence of the one that stimulates steroidogenesis. Similarly, other peptides contained in the large POLMC precursor peptide will be studied. Of special interest will be the large N-terminal portion of POLMC, the so-called 16K fragment. There is evidence in the literature which suggests that the

16 K fragment may have a role in potentiating the effects of ACTH on aldosterone and cortisol. All published studies have, however, been carried out in dispersed adrenal cells in vitro, a system which does not necessarily reflect physiological conditions. Finally, studies are planned to investigate the effects of these peptides on the function of the adrenal medulla.

Publications:

1. Gullner, H.-G., Kulakowski, E., Unger, R.H.: Somatostatin plays a role in the regulation of neurohypophysial vasopressin secretion. Annals of the New York Academy of Sciences 394: 142-146, 1982.

2. Gullner, H.-G., Yajima, H., Harris, V., Unger, R.H.: Kassinin: Stimulation of insulin and glucagon secretion in the rat. Endocrinology 110: 1246-1248, 1982.

3. Gullner, H.-G., Lakatua, D.J., Bartter, F.C.: Effect of inhibition of prostaglandin synthesis on urinary free dopamine excretion in women. Clin. Sci. 62: 209-213, 1982.

4. Gullner, H.-G., Harris, V. and Unger, R.H.: Kassinin and substance P stimulate somatostatin release in the rat. Clin. Sci. 61: 785-787, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01949-01 HE

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Further Studies on the Nature of Diabetes Insipidus Produced by Desoxycorticosterone in the Dog.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	David West	Guest Worker	HE NHLBI
	Hans-Georg Gullner, M.D.	Guest Worker	HE NHLBI
	John R. Gill, Jr., M.D.	Senior Investigator	HE NHLBI

COOPERATING UNITS (if any)
Dr. Gary L. Robertson, Section of Endocrinology, Department of Medicine, University of Chicago, Chicago, Illinois

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics Section

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	.5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Hypokalemia, polyuria, hypernatremia and overproduction of renal prostaglandins characterize prolonged treatment of dogs with desoxycorticosterone. The present studies indicate that the hypernatremia is associated with an increase in plasma arginine vasopressin despite a tendency for the osmotic threshold for vasopressin release to increase. The polyuria thus develops despite increases in plasma vasopressin and is unaffected by a further increase in plasma vasopressin produced by treatment with vasopressin. Treatment with indomethacin which corrects the overproduction of renal prostaglandins restores renal responsiveness to vasopressin and thereby corrects the polyuria, hypernatremia, and increased secretion of vasopressin. These findings suggest that in disorders such as primary aldosteronism, hypokalemia, hypernatremia and polyuria may be associated with an increase in circulating vasopressin and this may be a factor in the hypertension.

PHS-6040
(Rev. 2-81)

Project Description and Objectives:

In previous studies from this laboratory prolonged treatment with desoxycorticosterone (DOCA) produced potassium depletion, polyuria and hypernatremia and increased urinary prostaglandin(PG)E_2 in dogs on a high-sodium intake. As polyuria and hypernatremia were corrected either by potassium repletion which corrected the supranormal renal synthesis of PGE_2 or by indomethacin which inhibited its synthesis, renal water loss was presumably the result of an increase in renal PGE_2 synthesis, probably stimulated by potassium depletion. The present studies were designed to examine vasopressin release and the renal response to vasopressin during DOCA-induced polyuria and to evaluate further the role of prostaglandins in this phenomenon.

Methods Employed:

Five female dogs were housed in metabolic cages and given a constant diet that contained 115 mEq/day of sodium and 55 mEq/day of potassium. Water intake was ad libitum. After three or more days of equilibration, control observations were made from day 4 thru day 7 of the study; DOCA was then given from day 8 thru day 24 of the study. Pitressin tannate in oil (5U) was given every 12 hours on days 17,18,23 and 24 of the study. Urine was collected daily for determination of volume, sodium, potassium osmolality and PGE_2. Blood was drawn every two days for determination of sodium, potassium, osmolality and immunoreactive arginine vasopressin. On days 4 (control) and 11 (DOCA treatment) of the study, 5% sodium chloride was infused at 1 ml/min for 2 hours and then 5% dextrose solution was infused at 5 ml/min for 1 hour. Bloods were collected every 15 minutes for determination of sodium, osmolality and arginine vasopressin. The data were analyzed by plotting plasma arginine vasopressin as a function of plasma osmolality and determining the slope and intercept of the regression lines for the infusion studies performed during control and DOCA treatment; values for plasma osmolality at the points were the two regression lines intercepted the abscissa were taken as the rate of vasopressin release.

Major Findings and Significance:

Serum potassium, sodium, plasma osmolality and immunoreactive arginine vasopressin, urine volume, osmolality and PGE_2 during control, treatment with DOCA, and treatment with DOCA and indomethacin are shown in Table I. Plasma arginine vasopressin, urine volume, osmolality and PGE_2 during treatment with DOCA and DOCA plus pitressin tannate in oil are shown in Table II.

Table I

Regimen	Serum K^+ mEq/L	Serum Na^+ mEq/L	Plasma Osm mOsm/kg H_2O	Plasma Arginine Vasopressin pg/ml	Urine Volume ml/d	Urine Osm mOsm/kg H_2O	Urine PGE_2 ng/d
Control	4.6	144.6	298	6.1	480	1114	1350
DOCA	3.7	150.4	304	15.9	2250	254	2742
DOCA + INDO + Vasopressin	3.4	147.4	302	6.2	1290	615	714

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Table II

Regimen	Plasma Arginine Vasopressin pg/ml	Urine Volume ml/d	Urine Osm mOsm/kg H_2O
DOCA	15.9	1890	364
DOCA + Pitressin	24.3	2000	320

These results confirm previous observations that prolonged treatment with DOCA produces a rise in urine volume and a fall in urinary osmolality, and an increase in urinary PGE_2 presumably caused by the associated potassium depletion. The increase in water turnover was associated with an increase in serum sodium, plasma osmolality and plasma arginine vasopressin. Treatment with DOCA tended to increase the threshold or "set point" for vasopressin release from 299.3 to 304.5 mOsmol/kg H_2O but did not affect the rate of vasopressin release. Despite a tendency for the posterior pituitary to show a decreased responsiveness to osmotic stimuli, plasma vasopressin increased significantly ($P<0.01$) from 6.1 to 15.9 pg/ml pari passu with the increase in serum sodium from 144.6 to 150.4 mEq/L ($P<0.01$) and the increase in urine volume from 480 to 2250 ml/day ($P<0.01$). Treatment with pitressin tannate, although it produced a further increase in plasma vasopressin from 15.9 to 24.3 pg/ml, did not decrease mean urine volume (1890 versus 2000 ml/day) or increase urinary osmolality (364 versus 320 mOsmol/kg H_2O). The increase in water turnover was associated with an increase in urinary PGE_2 from 1350 to 2742 ng/day.

Treatment with indomethacin decreased urinary PGE_2 from 2742 to 714 ng/day and increased the renal responsiveness to vasopressin with the result that urine volume fell from 2250 to 1290 ml/day, urinary osmolality rose from 254 to 615 mOsmol/kg H_2O despite a fall in plasma vasopressin to 6.2 pg/ml, a value similar to control. Serum sodium also decreased towards control providing support for the notion that the fall in plasma vasopressin reflected increased renal responsiveness to vasopressin rather than a direct effect of indomethacin on vasopressin release. These improvements in water metabolism occurred without a change in hypokalemia and, presumably, were the result of correction of the overproduction of renal PG's. These results provide additional support for the hypothesis that DOCA-induced polyuria is mediated, at least in part, by an increase in the renal synthesis of PG's that oppose the action of vasopressin on the renal reabsorption of water.

Proposed Course of Study:

This study provides the new information that sodium-retaining steroids can lead to a sustained increase in the secretion of vasopressin. This suggests that a disorder such as primary aldosteronism that is characterized by an overproduction of a sodium retaining steroid, hypokalemia, hypernatremia and hypertension may also be characterized by an increase in circulating vasopressin that could contribute to the increase in blood pressure. This possibility will be explored in future studies.

Publications:

1. Dusing, R., Gill, J.R., Jr., Gullner, H.-G., Bartter, F.C.: The role of prostaglandins in diabetes insipidus produced by desoxycorticosterone in the dog. Endocrinology 110: 644-649, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01950-01 HE

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Study of the hypotensive action of the serotonin uptake blocker, CGP-6085.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Daniel J. Goldstein, M.D.	Expert	HE NHLBI
OTHER:	Elliot Kulakowski, Ph.D.	Staff Fellow	HE NHLBI
	Thomas G. Ropchak, B.S.	Biologist	HE NHLBI
	Harry R. Keiser, M.D.	Deputy Chief	HE NHLBI
	Walter Lovenberg, Ph.D.	Chief, Sect. on Biochem. Pharm.	HE NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension - Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	.8	.2

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The mechanism of action of the antihypertensive drug CGP-6085, a serotonin uptake inhibitor, was studied. The hypotensive effect of the drug is blocked by the opiate antagonist, naloxone, and by central serotonin depletion.

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PHS-6040
(Rev. 2-81)

Objectives: The hypotensive effect of clonidine in the spontaneously hypertensive rat (SHR) is reversed by naloxone. This drug also stimulates the release of β-endorphin from SHR brain slices. These data suggest that central endogenous opioids could play a role in the therapeutic action of clonidine. CGP-6085, a serotonin uptake blocker, has a significant hypotensive effect. It is well known that the hypotensive effect of some opioid peptides requires the integrity of the central serotonergic neurons. We started an investigation on the mechanism of action of CGP-6085 asking three basic questions:

(a) Are endogenous opioids involved in the hypotensive effect of CGP-6085?
(b) Is the hypotensive effect of CGP related to its known serotonin uptake blocking activity?
(c) Is the site of action of CGP-6085 central or peripheral?

Methods: Blood pressures of awake animals, Wistar Kyoto and SH rats, age matched, were measured by the tail-cuff method. CGP-6085 (10 mg/kg) was injected i.p. one hour before the administration of naloxone (2 mg/kg i.p.). Control animals were injected with saline. Para-chlorophenylalanine (PCPA), an inhibitor of serotonin biosynthesis, was administered three days before the injection of CGP 6085. After a dose of PCPA (350 mg/kg i.p.) the midbrain serotonin content of both WK and SH rats was reduced to 12 percent of the normal concentration, as measured by HPLC.

Results: Naloxone reversed the hypotensive effect of CGP-6085 in both WK and SH rats.

Treatment	Systolic Blood Pressure (Torr) ± S.E.M.	
	Wistar-Kyoto	SHR
Saline (n=4)	140 ± 4	160 ± 7
Naloxone (n=4)	.140 ± 5	158 ± 5
CGP-6085 (n=4)	100 ± 10*	125 ± 5*
CGP-6085 + Naloxone (n=4)	132 ± 4	150 ± 7

* $P < 0.05$

Depletion of central serotonin reduced the hypotensive effect of CGP-6085.

Pre PCPA (n=4)	-	178 ± 6
Post PCPA (n=4)	-	178 ± 7
CGP-6085 + PCPA (n=4)	-	171 ± 10

These results suggest, but do not prove, the activation of a serotonin-opioid axis by GCP-6085. If the drug, by inhibition of reuptake of synaptically released serotonin, would increase the serotonin concentration at the synaptic cleft and induce an enhanced release of opioids from midbrain neurons, in vitro experiments should demonstrate an enhanced release of opioids from midbrain slices incubated with CGP-6085.

Significance: Our results show that (a) serotonin is involved in the hypotensive action of CGP-6085, (b) a receptor blocked by naloxone participates in its mechanism of action, and (c) if naloxone acts by blocking opioid receptors, these might be peripheral.

Proposed course of project: The intracerebroventricular administration of naloxone will be done next. If centrally administered naloxone proves ineffective in suppressing the hypotensive effect of CGP-6085, it would confirm the inference of a peripheral mode of action of the opiate receptor blocker and hence, lead us to (a) the elimination of the peripheral sources of circulating opioids - the pituitary and the adrenal medulla and (b) the identification and measurement of the circulating peptide(s).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01951-01 HE

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Effect of Thyrotropin releasing hormone on the anti-hypertensive action of Captopril.

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Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	.75	.25

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Thyrotropin releasing hormone (TRH) reverses the hypotensive effect of the angiotensin converting enzyme inhibitor, Captopril, on the Goldblatt 2 kidneys 1 clip (2K-1C) model of renal hypertension in the rat. The TRH effect is central.

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Objectives: Angiotensin converting enzyme has been considered a possible physiological enkephalinase. If this were so its inhibition would lead to a longer half life of circulating opioid peptides. However, the opiate antagonist, naloxone does not reverse the effect of Captopril in the (2K-1C) hypertensive rat. TRH is a tripeptide that has many physiological functions in addition to that of regulating pituitary thyrotropin secretion. Among these effects is its ability to act in vivo as a partial antagonist of opiates and neurotensin. It has been shown recently that TRH is even more effective than naloxone in improving cardiovascular function and survival in experimentally induced endotoxic and hypovolemic shock, as well as in the improvement of neurological recovery after experimental spinal trauma. We decided to study the effect of TRH on the hypotensive effect of Captopril on the 2K-1C model of rat renal hypertension in order to answer the following questions:

(a) Is TRH effective in reversing the hypotensive effect of Captopril?
(b) If it does, is it a specific effect?
(c) Is the site of action of TRH peripheral or central?

Methods: The left renal artery of male Sprague Dawley rats was narrowed with a 0.2 silver clip. Three to four weeks later, once the hypertensive state was achieved, the animals were anesthetized with urethane and catheters implanted in the left carotid artery (to be connected to a pressure transducer and recorder) and in the right jugular vein (for the i.v. injection of drugs). For the intracerebroventricular (i.c.v.) administration of drugs, clipped and sham operated animals were stereotaxically implanted with steel cannuli in the lateral cerebral ventricles, 24 to 48 hours before the Captopril experiments.

Results: On 13 consecutive animals, TRH (2 mg/kg, i.v.) reversed the hypotension elicited by Captopril (2 mg/kg, i.v.).

	Mean Arterial Blood Pressure (torr) ± S.E.M. n=13
Pre-Captopril	135 ± 6.3
Post-Captopril	103 ± 8.4*
Post-Captopril + TRH	127 ± 8.4

* $P < 0.001$

This effect has some specificity, because TRH does not modify the hypotension induced by neurotensin (50 µg/kg, i.v.). Post-Captopril levels of blood pressure are reached again 10-30 minutes after the administration of TRH. Doses of TRH that do not have any effect i.v. (0.2, 2.0 and 20.0 µg/kg) produce dramatic increases of arterial blood pressure which last for 15 to 40 minutes. Our experimental results failed to support this hypothesis: i.e. CGP-6085 decreases, in a dose dependent fashion, the release of immunoreactive opioids from SHR midbrain slices.

Significance: These results extend the observations of Faden and Holaday about the profound cardiovascular effects of TRH, and open several options for future investigation. In addition, they suggest the desirability of designing TRH analogues which could have hypotensive effects.

Proposed course of project: We must study the specificity of this central effect, i.e. if it is able to reverse the hypotension induced by i.v. neurotensin, and determine the dose response curve of i.c.v. TRH. Future work will include study of the effect of subdiaphragmatic bilateral vagotomy on the i.v. TRH reversal of Captopril induced hypotension, as well as the evaluation of the role of the pituitary-thyroid axis in this response.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01952-01 HE

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Opiate antagonist activity in bee venom

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Hypertension-Endocrine Branch

SECTION
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1.0	.80	.20

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have detected, by the use of a bio-assay system, a naloxone-like activity (NLA) in whole, unfractionated venoms from the following varieties of bees: Vespula pennsylvanica, Vespula germanica, Vespula maculifrons, Polistes annularis, Dolichovespula maculata and Vespa craboro. We have been able to exclude some of the known autacoids that could mimic the effect of naloxone in our bioassay system.

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PHS-6040
(Rev. 2-81)

Objectives: Bee stings are painful. The pain could be due to the presence, in the injected venom, of substances that either (a) generate pain or (b) inhibit endogenous analgesic mechanisms. We are exploring the second alternative, which, if verified, could lead to the search for similar substance(s) in man.

Methods: We used the longitudinal muscle strip from the guinea-pig ileum, the bioassay preparation described originally by W D M Paton and M A Zar, for the characterization of the action of morphine and its reversal by naloxone and the NLA. Other bioassay preparations used were the rat and the mouse vas deferens (opiate receptors), the isolated guinea pig atria (acetylcholine and serotonin) and the parenchymal (lung) strip preparation (guinea pig) for histamine.

Results: So far, 6 of the 7 venoms studied have a NLA, as defined by the reversal of the morphine effect and the prevention of morphine action on the guinea pig ileum preparation. Now we have concentrated our efforts on the Vaspula annularis, which has an extraordinarily potent NLA. We have examined the following autacoids as responsible for the activity: acetylcholine, histamine, bradykinin and substance P. NLA is heat labile, and degraded by both pronase and trypsin. Preliminary experiments with HPLC fractionation have allowed us to partially purify the material from the bulk of the venom constituents.

Significance: If this NLA occurs also in man, it would demonstrate the existence of endogenous antagonist(s) of the enkephalin-endorphin system and open the way for the exploration of its physiological and physiopathological role in neurobiology and cardiovascular control.

Proposed course of project: We plan (1) to purify further and then to chemically characterize the NLA from the bee venom and (2) to continue the study of its pharmacological actions.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01953-01 HE

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Failure of Naloxone to Modify the Blood Pressure Action of Captopril

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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1.0	1.0	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The injection of naloxone after captopril had no significant effects on mean blood pressure in the 2 kidney, 1 clip (2K, 1C) rat model of renal vascular hypertension. We conclude that the antihypertensive response to captopril in the 2K, 1C hypertensive rat is not due to changes in an endogenous opiate system.

519

PHS-6040
(Rev. 2-81)

Objectives: Captopril, an angiotensin I converting enzyme (ACE) inhibitor, is capable of lowering blood pressure in situations where the renin-angiotensin system is not responsible for blood pressure maintenance. ACE also acts as an enkephalinase in the inactivation of enkephalin peptides. Thus, our hypothesis was that captopril may lower blood pressure by prolonging the action of endogenous enkephalins. If this is true, then naloxone, an enkephalin peptide antagonist, should block the antihypertensive action of captopril.

Methods: Nine male Sprague-Dawley rats had a silver clip (0.2 mm ID) placed on the left renal artery (2K-1C). Four to seven weeks following clipping, the rats were anesthetized with urethane 1.75 g/kg IP to cannulate the left femoral artery and vein. The arterial catheter was connected to a micropressure transducer and continuous recorder. Blood pressure was allowed to stabilize over 30 minutes. Each rat received captopril (2 mg/kg) IV followed in 5 minutes by naloxone (2 mg/kg) IV. Four 2K-1C male rats received naloxone (2 mg/kg) followed in 5 minutes by captopril (2 mg/kg) IV. Hemodynamic data were compared before and after treatment (Wilcoxon matched-pairs signed-rank test).

NIH dogs 24 hours after nephrectomy were either anesthetized or placed in a restraining jacket. The dogs had a femeral artery catheter connected to a pressure transducer.

Results: Captopril (2 mg/kg lowered mean blood pressure from 109 ± 8 torr to 80 ± 6 torr ($p<0.006$) in the 2 kidney, 1 clip rat (mean ± SEM). This dose was sufficient to block the action of 30 µg of angiotensin I administered intravenously. Naloxone (2 mg/kg) did not cause a significant change in the basal blood pressure of the rats. This dose inhibited the change in blood pressure induced by intravenous administration of either morphine sulfate (15 mg/kg) or leucine-enkephalin (100 µg/kg). Naloxone did not reverse the antihypertensive effects of captopril in the 2 kidney, 1 clip rat.

We have shown in anesthetized nephrectomized dogs that their blood pressure will decrease following administration of captopril, 2 mg/kg intravenously. The dog's blood pressure will increase following naloxone but the animal's level of anesthesia also decreases. When the restrained nephrectomized dogs are given captopril while awake, blood pressure did not change. Because the blood pressure did not decrease after captopril in the awake dog, this model is not useful in studying the effects of captopril on non-renin angiotensin blood pressure control systems.

Proposed course of project: We plan to test if naloxone will decrease the effect of Captopril in the awake 2K-1C rat model. Using awake animals would eliminate the possibility of anesthesia-induced changes in the endorphin system.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01954-01 HE

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October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Effect of Naloxone on Blood Pressure in Hypertensive Subjects

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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SECTION

Experimental Therapeutics

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1.5	1.2	.3

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The role of endorphins in the physiological regulation of systemic blood pressure and in the mechanism of action of clonidine, a centrally-acting antihypertensive agent, was investigated. Patients with essential hypertension controlled with clonidine did not change their blood pressure after administration of naloxone.

PHS-6040
(Rev. 2-81)

Objectives: Recent findings have suggested a role of endorphins as neuromodulators in the control of cardiovascular function on animal models. The hypotensive effects of two centrally acting antihypertensive agents, clonidine and methyldopa, have been reversed in spontaneous hypertensive rats by two opiate antagonists, naloxone and naltrexone (J. Pharmacol. Exp. Therap. 214: 203, 1980). We extended this study to hypertensive patients. We studied the effects of blockade of opiate receptors by naloxone on blood pressure and on the antihypertensive effects of clonidine.

Methods: The subjects were patients (age 18-55) with uncomplicated mild to moderate hypertension (BP 140/90 - 180/115 torr) untreated for at least 2 weeks. The patients' blood pressure was controlled for at least 2 weeks with either clonidine or clonidine and a diuretic (treated BP supine 140/90 or less) and then the patients were randomly assigned to receive either naloxone treatment the first week and placebo treatment the next or vice versa. Naloxone treatment consisted of three intravenous injections of naloxone (0.33 mg/kg, 0.66 mg/lg, and 2 mg/kg, respectively) 5 minutes apart. Blood pressure was recorded every 2 minutes for 1 hour after the last naloxone injection. A single 10 ml blood sample was collected 10 minutes after the last naloxone dose for catecholamine determination. Placebo treatment was similar except saline was substituted for naloxone.

Results and discussion: The acute administration of naloxone did not show any substantial effect on the blood pressure of subjects chronically treated with clonidine. The patients did not show any ill effects from the naloxone. There have been 4 patients treated with a total naloxone dose of 1 mg/kg and one patient received a total dose of 3 mg/kg.

Proposed course of project: We plan to administer naloxone to at least three more patients. We will then have a large enough sample to conclude if clonidine's antihypertensive action is reversed by opiate receptor blockade.

Publications: None.

ANNUAL REPORT
SECTION ON BIOCHEMICAL PHARMACOLOGY
HYPERTENSION-ENDOCRINE BRANCH
NATIONAL HEART, LUNG AND BLOOD INSTITUTE

During the past year, several new projects have been initiated. However, the major thrust of our research program remains directed toward an understanding of neurochemical systems. We have focused increasingly on phosphorylation systems, particularly those that are activated by Ca^{++} and calmodulin, and have established cultured cell systems to study the regulation of neurotransmitter synthesis. We have also devoted more time to the establishment of systems to study neuropeptides, particularly substance P and enkephalin-like compounds. We have continued to relate some of our fundamental studies to diseases, such as parkinsonism and stroke. In the following report we have divided the research into a number of subheadings, although many of the projects relate to more than one area.

I. Biogenic Amines

A major focus of the research in our laboratory has been devoted toward understanding the regulatory mechanisms of tyrosine and tryptophan hydroxylase. These enzymes are the key regulatory point in the enzymatic pathways for the synthesis of catecholamines and serotonin respectively.

Previously we had reported that in the striatum, tyrosine hydroxylase and the hydroxylase cofactor are highly localized within dopaminergic terminals. The concentration of the enzyme and its cosubstrate appears to be in the 100 uM range. The concentration of tyrosine (about 40 uM) is probably less than the total amount of enzyme. Based upon kinetic analysis one can determine that the majority (80%) of the enzyme is not active under the prevailing conditions within the terminal. The remaining 20% of the enzyme that is active is that which is phosphorylated. This phosphorylation process thereby appears to be a mechanism by which the neuron can immediately increase its rate of neurotransmitter synthesis. Based on our calculations it would appear that the active enzyme in the neuron in the resting state is nearly saturated with regard to tyrosine (40 uM), but that when a significant portion of the enzyme becomes activated (phosphorylated), the uptake of tyrosine into the neuron may become the rate-limiting step in the synthesis of dopamine. Evidence recently reported from other laboratories substantiates this concept since it has been shown that when the enzyme has been activated in vivo by dopamine receptor blockade, that rate of dopamine synthesis in the striatum is dependent upon the circulating level of the cosubstrate, tyrosine. These studies on the relationship of the enzyme tyrosine hydroxylase and its cosubstrate provide the necessary background for one of our long-term, objectives which is the ability to manipulate the rate of catecholamine synthesis. Since the majority of tyrosine hydroxylase is not phosphorylated, and this portion is not active because of its poor affinity for tetrahydrobiopterin, we have followed a strategy of trying to recruit some of the inactive enzyme by making more cofactor available. Our research has followed two directions: 1) attempting to establish systems to study its biosynthesis and, 2) attempting to administer tetrahydrobiopterin and active analogues to rats to enhance the rate of dopamine synthesis. With regard to the first approach, two systems have been used. Since the level of activity of the enzymes in the biosynthetic pathway for tetrahydrobiopterin appear to be very low, we have attempted to use the cultured mast cell and the cultured pineal gland both of which have relatively large amounts of cofactor. The mast cell system was difficult to evaluate since the rate of formation of pterin was proportional to cell

growth. The rat cultured pineal gland, however, has proven to be a more useful tool. It was possible to measure the formation of biopterin and dihydrobiopterin which were secreated into the medium. It was also possible to follow the incorporation of guanosine, the ultimate precursor, into the biopterin. However, total pterin content was unaffected by exogenous guanosine. Addition of sepiapterin to the medium resulted in significant increases in the oxidized forms of biopterin, but decreases in the reduced cofactor. In the course of these studies, we were also able to demonstrate that dihydrofolate reductase, a possible final enzyme in the formation of BH_4, was probably not involved in cofactor synthesis.

With little success in being able to maniplate cofactor levels through the modification of its synthetic pathway, a more immediate approach was to increase levels in brain by administering the compound directly to the animal. Initial studies in rats indicated that tetrahydrobiopterin penetrated the brain poorly when administered peripherally. 6-methyl tetrahydropterin, an active analogue of the cofactor, was administered to rats and found to penetrate the brain somewhat better but not sufficiently to cause a significant increase in dopamine turnover. We next attempted to administer tetrahydrobiopterin to monkeys that were fitted with spinal catheters so that continuous collection could be obtained. In this system, we found a relatively rapid increase in spinal fluid levels of the cofactor in all animals. However, the response in terms of increased dopamine or serotonin turnover was not large and was seen only in some of the animals. Based on the biochemical findings described above, it would appear that the cosubstrate tyrosine or typtophan must be coadministered if one is to see an increase in biogenic amine turnover. In a subsequent portion of this report we describe our initial experiments on the administration of the hydroxylase cofactor to man.

Tryptophan hydroxylase, which controls the rate of serotonin synthesis, is less well understood than the tyrosine hydroxylase system. This enzyme, which we determined to be regulated by a Ca^{++}-calmodulin dependent protein kinase, has never been substantially purified because of problems with stability. Two significant advances in this area have been made. A detailed study of the effect of reduced pterin on the oxygen and the thermal denaturation showed that this compound strongly stabilized the enzyme. In other studies, we have found that tryptophan hydroxylase, while not directly stimulated by calmodulin, binds to a calmodulin-Sepharose column in the presence of Ca^{++} and can be eluted with an EGTA solution. This provides an additional rapid step for the purification of the enzyme.

II. Calmodulin, Ca^{++} and Protein Phosphorylation

Protein phosphorylation reactions appear in many aspects of neuronal function. These include reactions that regulate presynaptic neurotransmitter synthesis as well as a variety of events both in the post-synaptic membrane as well as in the cytosol.

A variety of effector-specific protein kinases have been identified in brain tissue and, most recently, a cyclic nucleotide-independent protein kinase has been the subject of much interest. This particular protein kinase is stimulated by phospholipids in the presence of calcium. This phospholipid-sensitive kinase can apparently be distinguished from a calmodulin (CaM)-activated protein kinase on the basis of substrate specificity. However, it has not been demonstrated with certainty that the phospholipid- and CaM-activated protein kinase are distinct enzymes. We have demonstrated that the activation of protein kinase by phospho-

lipids can be mimickd by sodium dodecylsulfate (SDS), as well as the hydrophobic probe 8-anilino-1-napthalene-sulfonate (ANS), indicating that the phosphoipid effect on protein kinase represents a more general stimulation of enzyme activity than previously indicated.

Another enzyme, that may be identical to the above, is a pro-protein kinase. This brain protein has been isolated as described in the literature and we found it to be activated by proteolysis or Ca^{++} in the presence of 2-mercaptoethanol. Characterization of this enzyme suggests that it contains several types of subunits, including a calmodulin-like subunit.

We previously described a calcium- and calmodulin (CaM)-dependent protein kinase in synaptosomal cytosol. This kinase catalyzes the phosphorylation of several endogenous neuronal proteins. The protein kinase and major substrates bind to CaM-Sepharose in a calcium-dependent manner, and can be eluted by addition of EGTA to the eluent. This enzyme has presented difficulties for characteriztion because of its instability. It appears that there are two different mechanisms involved in the loss of enzyme activity. One is Ca^{++}-independent and results in loss of calmodulin sensitivity. The role of this enzyme remains to be determined, but it is of interest that chronic morphine treatment results in increased activity of this enzyme, particularly with regard to three endogenous proteins with molecular weights ranging from 50 to 60,000.

We have also initiated an investigation of protein kinase present in the extracts of PC-12 cells. Ca^{++}, however, appears to inhibit basal protein kinase activity and, while added calmodulin will overcome this inhibition, it is not apparent that there is a Ca^{++}-calmodulin dependent kinase in these cells. This system will, however provide an excellent opportunity to study protein phosphorylation in a neuronally related system.

The possible role of calmodulin as a component of the dopamine receptor has also been investigated. Of interest is the observation that manipulations which result in either increased or decreased sensitivity of the dopamine receptor in striatal membranes, results in a corresponding increase or decrease in the calmodulin content of the membranes. Thus, the specific role of calmodulin in the receptor complex is not known, but it clearly seems to modulate receptor sensitivity.

III. Neurotransmitter Release and Receptor Mechanisms.

In addition to our interest in neurotransmitters and the regulation of their synthesis in presynaptic areas, considerable effort has also been devoted to understanding how neurotransmitters are released and how post-synaptic elements respond to these transmitters. The rather large concentration of dopamine terminals and receptors in the striatum presents a good model for study. Incubation of striatal slices with dopamine, or apomorphine (all 10 uM) causes desensitization of dopamine-stimulated adenylate cyclase. The loss in responsiveness of adenylate cyclase appears to be mediated by prolonged occupancy of D-1 recognition sites. The selective D-2 receptor agonist LY 141865 did not elicit desensitization of dopamine-sensitive adenylate cyclase. These results were further confirmed by findings showing that haloperidol, but not sulpiride, blocked the desensitization elicited by dopamine. In dopamine-desensitized striatal slices the K_d for dopamine agonist binding was increased, while the activation of adenylate cyclase by NaF and cholera toxin was decreased. These results indicate that in dopmaine-

desensitized striatal slices the coupling of G/F protein with adenylate cyclase and dopamine recognition sites may be impaired. It was of interest to relate this work to human disease. One of the major theories of schizophrenia suggests an overactivity of central dopamine systems. Of interest are findings that, while the dopamine-dependent adenylate cyclase and calmodulin are not increased in brains from schizophrenics, the degree of stimulation of adenylate cyclase by F^- or GppNHp is significantly increased. This phenomenon was only observed in dopamine-rich areas and suggests that in these areas the regulatory subunit of the G/F protein allows an increased activity of adenylate cyclase.

In a related study, we found that cocaine binding sites were located on dopamine terminals and we characterized them. This binding site exhibits classical properties of a receptor and is partially sodium dependent. An endogenous inhibitor of cocaine binding has been partially characterized with the idea that this compound may represent an endogenous cocaine ligand.

Additional evidence has accrued supporting the concept that at least a portion of the neurotransmitter released from norepinephrine terminals in the heart is via a mechanism of active outward transport. Although some of the classical inhibitors of norepinephrine uptake inhibit the outward transport of this amine in heart ventricle slices, ion dependencies suggested that this outward transport is not simply the reverse of the uptake system. It would appear that such a mechanism would provide an attractive alternative or complement to the exocytotic concept of neurotransmission.

In regard to the cellular response to neurotransmitter activation of receptors, we have for several years studied the response of pinealocytes to B-receptor activation. In this system the pinealocyte responds to receptor stimulation with a dramatic induction of serotonin N-acetyl transferase. In the past year we have discovered that receptor blockade potentiates the response of the cells to norepinephrine by a factor of over 100. Similar potentiation can be observed with certain compounds that inhibit the reuptake of norepinephrine into nerve terminals. In the pineal glands from ganglionectomized rats receptor blockade alone is sufficient to cause an induction of the enzyme. These experiments have allowed us to develop new ideas of how different receptors on a single cell can interact to execute a response.

IV. Neuropeptides.

The role of peptides that appear to serve as neurotransmitters and neuromodulators is a subject of considerable interest in many laboratories. Our interest in these compounds arose because of their possible role in blood pressure regulation and their interaction with the more traditional neurotransmitters. In our last report we found that substance P turnover and metabolism in the striatonigral system could be manipulated by agonist and antagonists of the dopaminergic system. Our current studies have been devoted toward attempting to identify physiologic roles for this compound. In a series of experiments with an isolated working rat heart, substance P was found to significantly reduce coronary blood flow when present at concentrations of 10^{-6}-10^{-8} M. However, when an octapeptide (SP_{4-11}) that is a metabolite of substance P was tested, it was found to have an ED_{50} of 2×10^{-13} M. Subsequent studies indicated that proteolytic inhibitors could block the action of substance P, whereas these inhibitors did not affect the coronary vasoconstrictive response to the octapeptide. In another line of experimentation, the fetal and neonatal distribution of substance P in rat brain has

been examined. Of particular interest is the observation that by the 15th day of gestation there are substantial amounts of substance P in several gross brain regions, and that in most regions the concentration of substance P fell dramatically after birth. The role of this peptide in the developing brain is under continued investigation.

Research in other laboratories has revealed that the adrenal medullary cells contain a protein that has multiple copies of MET-enkephalin in its structure, and that there are significant amounts of fragments (enkephalin-like peptides) in the gland. The mechanism of release of these enkephalin-like peptides has been studied. Secretion of enkephalin-like peptides from adrenal glands into the circulation was studied in anesthetized dogs with indwelling cannulae in the lumbar adrenal vein. Splanchnic nerve stimulation causes a voltage-dependent increase of MET-enkephalin immunoreactive peptides and catecholamines in adrenal venous plasma. The effect of splanchnic nerve stimulation was mimicked by dimethylphenylpiperazinium and was blocked by hexamethonium. Thus the release appeared to be mediated by nicotinic receptors. In reserpenized dogs, storage of these peptides was unaffected, and they could be released by splanchnic stimulation. Following such stimulation and release there was a drop in arterial blood pressure which could be blocked by naloxone. This suggests that certain enkephalin-like peptides may be depressor in nature in anesthetized animals and may serve to counterbalance the pressor effects of released catecholamines.

Increasing evidence suggests that angiotensin functions as a neurotransmitter in the CNS. The synthesis of angiotensin II is catalyzed by a dipeptidyl carboxypeptidase (converting enzyme), which may also be involved in enkephalin metabolism. If the synthesis of A II or enkephalin is accomplished in nerve terminals, the activity of this enzyme should be present in synaptosomes. Fractionation of tissue homogenates on a discontiuous sucrose gradient allows the preparation of highly purified synaptosomes. Of interest is the finding that the specific activity of dipeptidyl carboxypeptidase was higher in the synaptosomal fraction than in any of the other subcellular fractions, suggestng a specific role for this enzyme in the synthesis of this neurotransmitter peptide.

V. Blood Pressure Control

Both the central and peripheral nervous systems are extremely important in controlling blood pressure in mammals under normal and possibly under pathological conditions. Several of the neurotransmitter systems we are investigating appear to participte in these control mechanisms.

All of the cell bodies for the epinephrine neurons are strategically located in the brain stem near some of the blood pressure control centers. While their role in blood pressure control remains undefined, we have previously shown that spontaneously hypertensive rats have increased numbers of these cell bodies. This is also reflected in an increase in the amount of the enzyme phenylethanolamine-N-methyl transferase. In an attempt to determine whether these specific neurons participate in the baroreflex action, normotensive and spontaneously hypertensive rats had unilateral caroted artery ligations performed in a manner that would result in a marked pressure differential on the barorecepters. Six hours after surgery, the activity of PNMT was measured in the left and the right C_1 and C_2 regions. In sham-operated animals the enzyme activity exhibited no laterality and was about 25% higher in SHR than in normotensive animals. However, in both C_1 and C_2 regions in both hypertensive and normotensive rats there was a significant in-

crease in activity contralateral to the artery ligation. While much remains to be learned, this is the first evidence that the epinephrine system in brain responds to or participates in the baroreflex.

Another area of continuing study relates to the role of the central serotonin system in blood pressure control. We previously reported that direct stimulation of certain groups of serotonin neurons in the brain results in major increases in blood pressure, and pharmacological studies have identified the active component as serotonin neurons. Recent work has been directed at identifying the pathway eliciting this response. Microinjection of serotonin directly into the nucleus tractus solitarius produced a dose dependent increase in arterial blood presure of anesthetized rats. The serotonin antagonists BOL and metergoline significantly attenuated the serotonin pressor effect and the serotonin uptake inhibitor fluoxetine significantly enhanced the magnitude of the pressor response. Electrical stimulation of the dorsal raphe nucleus or microinjections of serotonin into the preoptic regions of the hypothalamus produced a transient rise in arterial blood pressure of both spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) controls. These effects can be attenuated by metergoline. These studies suggest that the information ascends to higher centers before it returns to the areas that control sympathetic outflow.

It appears, however, that, in addition to the pressor serotonin pathway, neurons using this transmitter may also be involved in depressor systems. A new drug CGP 6085 A that inhibits serotonin uptake was found to exhibit a powerful depressor response when administered to either hypertensive or normotensive rats. Studies to date are consistent with the depressor effect being related to inhibition of serotonin uptake. Our tentative conclusion is that neurons utilizing serotonin as a neurotransmitter participate in both pressor and depressor systems. Evaluation of CGP 6085 A as a hypotensive agent is continuing.

Another approach to understanding blood pressure control was to utilize our experience with protein phosphorylating systems to examine protein kinase and phosphorylation patterns in the vasculature of the spontaneously hypertensive rats. A careful characterization of the cAMP and cGMP protein kinases under optimized conditions for substrate saturation revealed no major differences in the kinase activity in the aorta from hypertensive or normotensive rats. This contrasts with several reports in the literature indicating either higher or lower kinase activity. However, conditions of incubation were not optimized in these earlier reports.

VI. Research Relating to Disease States

In addition to our studies on blood pressure control, certain projects appear to have fairly direct relevance to particular diseases. One such project is derived from our studies on the hydroxylase cofactor several years ago. We first reported that the content of this compound in the CSF was inversely related to age in both normal and parkinsonian patients and that the mean content in the latter patients was about 50% that of the age-matched controls. This probably reflects the known loss of dopaminergic neurons in this disease. The possibility that inactive tyrosine hydroxylase could potentially be recruited by increasing hydroxylase cofactor levels in brain and thereby increasing dopamine sythesis at the appropriate location, led us to propose administration of tetrahydrobiopterin to patients with parkinsonism. During the past year this has been accomplished. In collaboration with researchers in NINCDS, the first patients have been given the

compound. Researchers in Japan and Switzerland have also pursued this approach. No dramatic improvements in neurological symptoms were observed in any laboratory, although the results form Japan and Switzerland indicate that certain patients had neurological improvement. The results are currently sufficiently encouraging to induce further experimentation. It should be noted that neurological symptoms in children with a genetic defect in hydroxylase cofactor synthesis respond dramatically to administration of this compound.

In studies with the stroke-prone substrain of spontaneously hypertensive rats, it appears that genetic and environmental factors interact to enhance the likelihood of cerebro-vascular lesion. In recent experminets we have found that increasing the protein content of the diet from about 17% to 22% reduces the incidence of cerebral lesions in these animals from about 85% to about 30% at 9 months of age. This effect appears to be attributable to the protein content, since there appears to be no difference in other components of the diet. Growth rates and blood presures are about the same on either diet. These observations are consistent with preliminary epidemiologic studies done in Japan, in which population groups with decreased protein uptake exhibited a significantly higher incidence of death from stroke. Studies are continuing on a collaborative basis with researchers in Japan, in an attempt to identify the protective mechanism that increased dietary protein has on the incidence of stroke.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01843-09 HE

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Mechanisms of Storage, Uptake and Release of Norepinephrine in Adrenergic Nerve Endings

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Donald R. Bogdanski Pharmacologist HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Two phases of the overall project described in previous years work were rounded out for publication. The choline+ and Ca2+ stimulated secretion of 3H-NE in adrenergic nerve endings in rat heart ventricle slices was inhibited by ATP and other triphosphorylated nucleosides. A new aspect of this problem was begun. The effect of ATP was antagonized by N-ethyl-maleimide, chlorpromazine and 2,4-DNP. The inhibiton of secretion by the transport inhibitors, cocaine and desipramine, was Na+-dependent and antagonized by K+. The antagonism by K+ could explain why neurotransmission is not strongly, if at all, antagonized by transport inhibitors. Evidence suggests that the outward transport of NE during Ch+-Ca++ stimulation is mediated by a different mechanism than that which mediates the uptake of NE.

PHS-6040
(Rev. 2-81)

Objectives: To accumulate evidence that the norepinephrine stored in synaptic vesicles in the adrenergic nerve endings in rat heart can be secreted by a transport mechanism which translocates the NE mobilized in synaptic vesicles fused to the plasmalemma of the nerve ending. The morphological unit of fusion includes the NE transporting mechanisms of both the plasmalemma and vesicle membranes. The specific objectives of the past years work was to complete a phase of the studies reported in previous years. These studies include the effects of ATP and transport inhibitors on the inhibition of Ch^{+}-Ca^{++} stimulated release.

A new phase of these experiments was begun. The efect of some metabolic inhibitors as antagonists of the ATP-induced inhibition of Ch^{+}-Ca^{++} stimulated release was tested. The objectives of these studies was to establish similarities between the actions of ATP on Ch^{+}-Ca^{++} stimulated release and on the release of NE in isolated vesicles. Experiments were run to determine whether uptake and release were mediated by reverse processes of the same mechanism.

Methods: The methods used for the studies of choline and Ca^{++} (Ch^{+}-Ca^{++}) stimulated neurosecretion of ^{3}H-NE in adrenergic nerve endings of the rat heart were previously described in detail. The methods used for the study of uptake by rat heart ventricle slices were also reported previously.

Major Findings: Experiments during the past year completed the studies showing the Na^{+}-dependency of the neurosecretion inhibiting effects of cocaine and desipramine. These studies were described in detail for last year's report. The experiments were performed as previously described. In the experiments for the past year, the effect of the transport inhibitors was tested during the 70th to the 90th min of Ch^{+}-Ca^{++} stimulation, at which time the rate of efflux was maximal. The tissues were transferred to a Na^{+} enriched medium, containing inhibitor, either Krebs-bicarbonate (KRB) or Ch^{+}-Ca^{++} with Na^{+} added to a concentration of 76 to 86 mM. The tissues were incubated with inhibitor for an interval of 20 min, then they were transferred to a fresh, standard Ch^{+}-Ca^{++} medium for studying neurosecretion as usual. The results of experiments were as follows: (1) Sodium^{+}, by itself, inhibited Ch^{+}-Ca^{++} stimulated release, but the effect disappeared during the subsequent incubation of slices in standard Ch^{+}-Ca^{++}. (2) Either cocaine or desipramine in the Na^{+}-enriched media inhibited the neurosecretion. This inhibition outlasted the presence of Na^{+} in the incubation media. (3) The persistent effect of the inhibitors was best observed when the tissues were exposed to the inhibitors during the final 20 min of the 90 min (physiologically equilibrating) preincubation of the slices in KRB. Rapid secretion was not observed during the subsequent 140 min of incubation, indicating that the effects of desipramine were not readily reversible in the Ch^{+}-Ca^{++} medium. (4) Transport inhibitors added to the incubation medium without supplemental Na^{+} failed to inhibit Ch^{+}-Ca^{++} stimulatied neurosecretion.

The effects of K^{+} (66 to 76 mM) were also studied and the results are presented as follows: (1) K^{+} stimulated the release of ^{3}H-NE whether the K^{+} was introduced into the medium during the preincubation in KRB, the Ch^{+}-Ca^{++} medium before the onset of rapid release or during the phase of rapid release. (2) The transport inhibitors had no effect on the neurosecretion evoked by K^{+}. (3) The release stimulating effect of K^{+} was generally followed by a prolonged phase of slow release, even if K^{+} was still present in the medium. (5) If K^{+} was introduced during the phase of rapid Ch^{+}-Ca^{++} neurosecretion, however, the K^{+} did not induce a secondary inhibition of neurosecretion. (6) Thus, it was possible to determine that K^{+} interfered with the establishment of Na^{+} dependent, desipramine

(or cocaine) induced inhibiton of neurosecretion. The K^+ was included in the media during the 70th to 90th min of preincubation, or, incubation in Ch^+-Ca^{++}, together with the Na^+ and inhibitor.

It was tentatively concluded that the outward transport mechanism was similar, in some respects, to the uptake mechanism. Transport in both directions is inhibited by transport inhibitors, and is at least partially dependent upon the presence of Na^+ and inhibited by K^+. The effect of electrolytes on the actions of transport inhibitors appears to represent a new aspect of the actions of these drugs. The attachment of the inhibitor to its receptor appears to be under the control of monovalent electrolytes - increased by Na^+ and decreased by K^+. Those characteristics parallel the control exerted by these same electrolytes over the apparent K_m (affinity) between the uptake carrier and transmitter amines. Thus, there exists a fairly wide range of parallels between the mechanism(s) of uptake and outward transport. An effort was, therefore, made to determine whether a similar electrolyte dependency could be shown for the effects of the transport inhibitors on the uptake mechanism. It was desired to determine whether there was more complete parallels between outward transport and uptake, hence, the first experiments were similar to the neurosecretion experiments. That is, the tissues were preincubated 90 min in KRB and then transferred to the Ch^+-Ca^{++}. However, prolonged exposure of the slices to Ch^+-Ca^{++} inhibited the uptake of ^{3}H-NE during subsequent incubation of the slices in KRB. Therefore, neither the Na^+ requirement for uptake, nor the inhibitory effect of K^+ on transport inhibition, could be tested in this way. This negative finding appears to differentiate the processes of uptake and Ch^+-Ca^{++} stimulated efflux into 2 different mechanisms. One mechanism functions under experimental conditions which inactivate the other.

The K^+ effect as an antagonist of desipramine induced inhibition of Ch^+-Ca^{++} stimulated release might possibly offer some explanation for the failure of transport inhibitors to block synaptic transmission. This explanation would be related to the nerve depolarization and the re-distribution of electrolytes that are a part of synaptic transmission. These physiological actions would tend to antagonize the attachment of the transport inhibitors to their receptor involved with outward transport, if outward transport mediated synaptic transmission. However, it is not yet known whether the transient depolarizations, and the accompanying, transient ion movements that characterize nerve activity would have a similar inhibitory effect on inhibitor attachment as the steady and prolonged effects of high concentratons of K^+. Additional studies in this area of study are being considered.

The energy requirement for the ATP induced antagonism of Ch^+-Ca^{++} stimulated neurosecretion was studied with the aid of non-energy-conserving synthetic analogs of ATP; which are: AMP-PCP and AMP-PNP. These compounds also test the possibility that ATP antagonizes neurosecretion by binding Ca^{++}. These compounds bind Ca^{++} as tightly as ATP binds Ca^{++}. Hence, it could be expected that their action would parallel the action of ATP in all respects, if Ca^{++}-binding were the mechanism of action of all the nucleotides. However, ATP antagonized Ch^+-Ca^{++} stimulated neurosecretion almost immediately, whereas the synthetic analogs of ATP had almost no effect until more than 20 min had elapsed. The neurosecretion antagonizing effects of all three compounds were similar, if not equal, after 60 min had elapsed.

Other evidence also suggests that Ca^{++} binding was not the mechanism of action of ATP. Chelation of Ca^{++} by EDTA temporarilly antagonized the Ch^+-Ca^{++}

stimulated neurosecretion, but subsequently, the neurosecretion became stimulated. Thus, the effect of ATP and the other energy conserving nucleotides appears to be distinct from that of the synthetic analogs of ATP and chelators.

The action of ATP as an antagonist of Ch^{+}-Ca^{++} stimulated neurosecretion differs from that of adenosine and its partially phosphorylated nucleotides as antagonists of nerve-stimulated release of NE. These compounds have a small effect on the Ch^{+}-Ca^{++} stimulated nuerosecretion. Moreover, theophylline antagonizes their effect on nerve-stimulated release, but has no effet as an antagonist of ATP-induced inhibition of Ch^{+}-Ca^{++} stimulated neurosecretion. Thus, the neurosecretion inhibiting effect of ATP, and, therefore, the Ch^{+}-Ca^{++} stimulated neurosecretion, does not appear to fall into any existing categories of physiological and pharmacological actions except for amine transport at the vesicle membrane.

A study was begun which was intended to investigate the metabollic requirements for the retention of NE by the synaptic vesicles in peripheral adrenergic nerve endings in rat heart. Previous investigations along these lines have been reported and published. The present work extends these previously reported findings and intends to demonstrate vesicular metabolic process as reflected in Ch^{+}-Ca^{++} stimulated neurosecretion. Since these studies are in a preliminary state, they will be described briefly.

The ATP induced inhibition of Ch^{+}-Ca^{++} stimulated release was antagonized by N-ethylmaleimide (NEM). This inhibitor of Mg^{++}-ATPase would be expected to antagonize an effect of ATP if the effect was mediated by the activation of Mg^{++}-ATPase. The enzyme is currently thought to mediate the transport (uptake) of NE by an amine pump in the vesicle membrane. However, NEM also depletes the NE in slices incubated in KRB. Its actions are not specific for Mg^{++}-ATPase, therefore, conclusions drawn from it are limited.

Chlorpromazine also antagonized the ATP inhibition of Ch^{+}-Ca^{++} stimulated release, but it, too, released NE in the slices incubated in KRB. The dissipator of stored energy, 2,4-dinitrophenol, appeared to show some degree of specificity as an antagonist of ATP induced inhibition of Ch^{+}-Ca^{++} stimulated release.

General Summary: It appears that the mechanism for outward transport differs from the mechanism for transport in its response to prolonged exposure to Ch^{+}-Ca^{++}. It is concluded that, either different mechanisms mediate the transport of NE in each direction, or, that a damaged carrier mechanism will transport the amine in one direction but not the other It is also a fact that so-called outward transport has not yet met the criterion of saturability, necessary for the demonstration of carrier mediation.

Significance to Biomedical Research and Institute Programs: The storage and release of nerve transmitters is fundamental to the functioning and existence of complex organisms, such as man. Our studies have been directed towards describing a model neurosecretory system. This system becomes locked into a status secretorius, which can deplete the nerve ending of its store of NE and utilizes a channel of release that is different from exocytosis. Hence, this mechanism can possibly mediate the normal or pathological release of neurotransmitter. The experiments in this laboratory are intended to describe the mechanism of this release.

Proposed Course of Project: The immediate objectives in the near future are to study the effects of metabolic inhibitors on the ATP induced antagonism of Ch^{+}-

Ca^{++} release as described in a previous report. We hope to consolidate the hypothesis that the effect of extracellular ATP is indicative of its access to the vesicle membrane from the cell exterior. This finding would support the hypothesis that Ch^{+}-Ca^{++} stimulated secretion represents an outward transport of NE from vesicles fused to the plasma membrane to form a structure that contains amine transporting mechanisms of both membranes. This new structure does not proceed to fission and exocytsis.

Publications:

Bogdanski, D. R., Evidence for the Outward Transport of Norepinephrine in Synaptic Vesicles Attached to the Plasma Membrane, Neuropharmacology, 1982, In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01846-08 HE

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Molecular Biology of Chemoreceptor Regulation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Ingeborg Hanbauer Pharmacologist HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Acute hypoxia releases dopamine from glomus cells without altering the norepinephrine stores in carotid body. A similar pattern of changes in catecholamine content is elicited by the injection of muscarinic receptor agonists. Methylatropine prevents the release of dopamine elicited by methacholine and by acute hypoxia, suggesting that a cholinergic muscarinic receptor may mediate dopamine release. In contrast, long lasting hypoxia increases dopamine and norepinephrine content in carotid body. The increase of catecholamine content is elicited by induction of tyrosine hydroxylase, hypertrophy and possibly also hyperplasia of glomus cells. Carbohydrate-active steroids increase the catecholamine content in carotid body suggesting that these steroids may exert trophic functions during long-lasting hypoxia.

PHS-6040
(Rev. 2-81)

Objectives: For several decades the understanding of chemoreceptor function of the carotid body has been based solely on electrophysiological and morphological findings. Only recently have substantial neurochemical and pharmacological contributions brought new knowledge on the chemical transduction of chemosensory stimuli and on synaptic contacts between glomus cells and carotid sinus nerve endings. Dopamine was shown to be the major neurotransmitter present in glomus cells, whereas norepinephrine is primarily located in sympathetic nerve endings in the vascular bed of this organ. Recently, the presence of peptide hormones has been described in glomus cells and carotid sinus nerve endings. The scope of this project is to determine the biochemical mechanisms underlying the chemoreceptor response in carotid body. It is of interest to determine the role of catecholamines and polypeptides as primary transmitters or as co-transmitters in the chemoreceptor function of carotid body.

Methods: Rats are exposed to hypoxia (10% O_2 in 90% N_2) for short-term or long-term periods. In some studies transection of the carotid sinus nerve or superior cervical ganglionectomy was performed 5 to 7 days before the experiment. The content of dopamine, norepinephrine and their metabolites was measured by a mass fragmentographic procedure using deuterated analogues as internal standards.

Major Findings:
Neurochemical changes of putative neurotransmitters during short-term hypoxia. Exposure of rats to 5% O_2 in 95% N_2 elicits a drop in pO_2 from 117 to 35 torr and causes a time-dependent release of dopamine from glomus cells. A similar hypoxic stimulus failed to change the content of norepinephrine or acetylcholine in carotid body. Extensive work on denervated carotid body showed that neither the innervation of the carotid body by the carotid sinus nerve nor by the ganglio-glomerular nerve played a role in mediating the dopamine release. Studies on the effect of methacholine showed that this drug elicited a similar decrease of dopamine content in carotid body without causing a change in norepinephrine content. Methylatropine was able to partially prevent the depletion of dopamine elicited by short-term hypoxia or by injection of methacholine. These findings suggest that muscarinic cholinergic receptors may mediate the release of dopamine during acute hypoxia.
Biochemical aspects on neurotransmitters during long-lasting hypoxia. Long-term exposure to 10% O_2 in N_2 elicited a significant increase in dopamine content already after two days of exposure, but failed to cause a change in norepinephrine content. Exposure to hypoxia for one week elicited an increase in norepinephrine content also. Exposure to 10% O_2 in N_2 for 4 weeks elicited a 14-fold increase in dopamine content and a 12-fold increase in norepinephrine content. This gradual increase in catecholamine content in carotid body was attributed to induction of tyrosine hydroxylase in carotid body.

Experiments carried out with carbohydrate-active steroids showed that dexamethasone causes an induction of tyrosine hydroxylase and dopamine-beta-hydroxylase and leads to an increase in dopamine and norepinephrine content in carotid body. Similar to the effects of long-term hypoxia the effects elicited by dexamethasone were not curtailed by denervation of the carotid body. The regulation of catecholamine levels and synthesizing enzyme activity by carbohydrate-active steroids may be an important mechanism involved in acclimatization to hypoxic conditions.

Significance to Biomedical Research and Institute Programs: The arterial chemoreceptors play an important role in the respiratoy and circulatory response to

hypoxia, hypercapnia or metabolic acidosis. One of the main problems in cartoid body function is to understand the mechanism whereby chemosensory impulses are initiated. To analyze the events occuring at the receptor level physiologically and biochemically, it is necessary to know how the carotid body is structurally organized and the type of specific neurotransmitters involved in the chemosensory response. Our studies reveal that catecholamines present in the glomus cell are involved in the chemoreceptor response during short- and long-lasting exposure to hypoxia.

Proposed Course of Project: The following experimental approach is planned to improve our understanding of the biochemical mechanisms involved in chemoreceptor function. Measurements of peptide hormones including met-enkephalin-like peptides, vasoactive intestinal peptide and substance P will be studied during short- and long-term hypoxia.

Publications:

Hellstrom, S., Hanbauer, I., Commissiong, J., Karoum, F. and Koslow, S.: Role and Regulation of catecholamines in carotid body. In: Dynamics of Neurotransmitter Function, Raven Press, New York (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01847-08 HE

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Functional Role of Calmodulin in the Regulation of Neurotransmitter Receptors.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ingeborg Hanbauer	Pharmacologist	HE NHLBI
OTHER:	Maurizio Memo	Guest Scientist	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

CHECK APPROPRIATE BOX(ES)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Calmodulin is a regulatory component in the function of striatal dopamine receptors, because it controls the synthesis, metabolism and action of cyclic AMP. The calmodulin content in striatal membranes increases during dopamine receptor supersensitivity elicited by cocaine. In addition, the responsiveness of adenylate cyclase to dopamine stimulation and the apparent number of dopamine recognition sites are increased. Cocaine failed to modify the function of the GTP-binding protein which couples the dopamine recognition sites to adenylate cyclase. The present results suggest that cocaine may interact with postsynaptic membrane proteins and thereby increase the availability of membrane-adenylate cyclase.

538

PHS-6040
(Rev. 2-81)

Objectives: Dopamine receptors like many other post-synaptic receptors function as supramolecular entities where receptor activity is generated by the interaction of a number of specific membrane proteins. These proteins which have been more or less characterized are imbedded in the membrane lipid bilayers and include proteins responsible for transmitter recognition, a coupling protein, a transducer usually adenylate cyclase and modulator proteins for adenylate cyclase such as calmodulin. Calmodulin may regulate the function of dopamine receptors at various steps. It may regulate the coupling of the recogition site to adenylate cyclase or it may directly modulate adenylate cyclase activity. Its action also extends to the regulation of phosphodiesterase and protein kinase which may play a role in the down-regulation of the dopamine receptor.

The present project was carried out to evaluate the participation of calmodulin in the regulaton of striatal dopamine receptor-linked adenylate cyclase.

Methods: Rat striatal slices were first preincubated in Krebs-bicarbonate buffer, pH 7.4, then drugs were added which were shown to modify dopaminergic transmission or cyclic nucleotide function and the incubation continued for various periods of time.

Adenylate cyclase activity was measured in homogenates prepared from striatal slices as described by Clement-Cormier et al., [Proc. Natl. Acad. Sci. 71, 1113-1117, 1974]. Specific ligand binding to dopamine receptors was carried out using ^{3}H-N-Propylnorapomorphine and ^{3}H-spiroperidol [Creese et al., Europ. J. Pharmac., 56, 411-412, 1979; Burt et al., Science, 11, 96, 326-328, 1977]. Calmodulin content in cytosol and membrane extracts was measured by enzyme-linked immunosorbent assay (Hanbauer et al., N.Y. Am. Acad.Sci., 356, 292-303, 1980.).

Major Findings: Calmodulin appears to be involved in the regulation of striatal dopamine receptors during supersensitivity elicited by cocaine. Injection of cocaine into rats increased the calmodulin content in striatal membrane preparations. The susceptibility of striatal adenylate cyclase to stimulation by dopamine was increased 30 to 60 min following injection of cocaine into rats (20 mg/kg ip) or after incubation of striatal slices with cocaine (10^{-6}M). In contrast the response of adenylate cyclase to stimulation by NaF (1-10 mM) or cholera toxin (10-100 ug/ml) was similar in saline or cocaine treated rats. These findings indicate that the increased responsiveness of adenylate cyclase to dopamine stimulation is not elicited by an increased availability of coupling protein, but appears to be facilitated by an increase of membrane-bound calmodulin content.

In striatal slices incubated with cocaine, the B_{max} for specific binding of spiroperidol or N-propylnorapomorphine was significantly increased, which is in line with supersensitivity of dopamine-sensitive adenylate cyclase elicited by chronic treatment with neuroleptic or transection of the nigra-striatal fiber-bundle.

Significance to Biomedical Research and Institute Programs: Considerable attention has been paid to neurotransmitter receptors and their regulation in various tissues and various pathological states of the nervous system. Since receptor activity can be viewed to be linked by second messengers (cyclic AMP) to biochemical processes in neuronal membranes, our studies on the heteromolecular complex of the striatal dopamine receptor may help to clarify the mechanism of action of

neuroleptic and narcotic drugs and provide insight into mental disorders.

Proposed Course of Project: The function of striatal calmodulin in dopamine receptor supersensitivity elicited by cocaine or neuroleptics will be further studied with respect to interaction with other receptor components. Future studies are planned to determine whether cocaine increases the calmodulin content in striatal membranes and consequently causes dopamine receptor supersensitivity or whether other mechanisms linked to specific binding of cocaine or neuroleptics to membrane proteins may be operative. The action of cocaine on phosphorylation or dephosphorylation of membrane proteins will be examined, because these mechanisms could be crucial in modifiying the affinity of a calmodulin-binding protein.

Publications:

1. Memo, M., Pradhan, S. and Hanbauer, I., Cocaine-induced supersensitivity of striatal dopamine receptors: role of endogenous calmodulin. Neuropharmocology, 20, 1145-1150, 1981.

2. Hanbauer, I. and Memo, M., Long-term modulation of dopamine receptor sensitivity: participation of calmodulin. In: Apomorphine and other dopaminomimetics, Vol 1, Basic Pharmacology (eds. G.L. Gesso and G.V. Corsini), pp 171-177, Raven Press, New York, 1981

3. Costa, E., Hanbauer, I. and Memo, M., Calmodulin and dopamine receptor regulation, In: Advances in Pharmacology and Therapeutics II (eds. H. Yoshida, Y. Hagihara and S. Ebash), Vol 4, pp 147-155, Pergamon Press, Oxford and New York, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01850-13 HE

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Biochemistry of the Spontaneously Hypertensive Rat

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Walter Lovenberg	Chief, Sec. Biochem. Pharm.	HE NHLBI
OTHER:	M. Fujiwara	Visiting Fellow	HE NHLBI
	Yukio Yamori	Guest Worker	HE NHLBI
	Hisao Tanase	Guest Worker	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.8	0.2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The role of hyperplasia and hypertrophy of vascular tissue in the development of increased blood pressure in rats has been studied. Genetic studies revealed that in the spontaneously hypertensive rats a single gene accounted for nearly 60% of the observed increase in blood pressure. Further examination of a number of strains of rats with varying blood pressures revealed that heart and vessel enlargement were due only in part to the increased pressure to which they were exposed, but were significantly affected by genetic factors unrelated to those regulating blood pressure. These conclusions were drawn from studies on DNA and RNA metabolism as well as physical measurements. In other types of experiments the expression of the apparent vascular hypertrophy was studied by the measurement of the rates of amino acid incorporation into various protein fractions of brain microvessels prepared biochemically. In contrast to earlier studies on mesenteric vessels, brain microvessels from spontaneously hypertensive rats primarily exhibited an increased rate of amino acid incorporation into the collagen fraction.

541

Objectives: The underlying causes of essential hypertension remain to be discovered. The increased peripheral resistance that is usually observed may result from sympathetic overactivity and/or altered physical characteristics of the resistance vessels. The objectives of the current research is to learn more about the genetic regulation of heart and vascular structures and to determine whether altered vascular structure preceeds or is caused by the increase in blood pressure. Rat models of hypertension have been the experimental animals and prior studies from our laboratory suggested that sympathetic nerves, in addition to maintaining vascular tone, also have a trophic effect on the rate of protein synthesis in small resistance vessels. One of the major objectives of our laboratory continues to be an understanding of how the CNS executes its blood pressure regulatory role in the periphery

Methods: Animals were obtained from the NIH small animal section and their blood pressures were monitored by tail cuff plethsmography. Cerebral microvesels were prepared by previously described standard methods and were usually taken at a fixed time after the I.V. administration pulse of radioactive amino acid. Following preparation of the microvessels, the proteins were extracted and divided into collagen (hot TCA soluble) and non collagen fractions. For genetic studies, 23 strains of inbred rats were used and blood pressures monitored as described above. At various ages, hearts and aortas were removed, weighed, physical dimensions determined and in certain experiments analyzed for RNA and DNA content.

Major Findings: In prior years we had reported a sympathetic dependent increase in non-collagen protein synthesis in small peripheral blood vessels. In the current studies we were able to measure the incorporation of amino acids into protein from brain microvessels. With this approach, however, we found that the increased vascular protein synthesis in this tissue was attributed mainly to collagen protein. We are currently attempting to resolve this difference, although changes in brain may largely reflect a result of hypertension, whereas peripheral vessels, which serve a resistance role, may exhibit early changes in contractile protein synthesis.

The heart and aorta weights in twenty three strains of rats and the four way cross generations among the M520/N, SHRSP/N, SHR/N and WKY/N strains were investigated in relation to their blood pressure in an attempt to characterize cardiovascular enlargement (increased weight of heart and aorta) from a genetic aspect. The distribution of blood pressure in these strains at ten weeks of age was clearly divided into hypertensive and normotensive groups. In the hypertensive group, heart weight increased in proportion to blood pressure. In contrast, there was no relationship between blood pressure and heart weight in the normotensive group in spite of large strain differences in heart weights. The result of variance analysis exhibited a significant strain difference in heart weight, and the degree of genetic determination was estimated to be 65-75 percent. A similar genetic influence was apparent for normotensive strains excluding hypertensive strains. The distribution of blood pressures in the four way cross generation showed the segregation of three phenotypes consisting of normotensive, intermediate and hypertensive groups. A large variability was seen in heart weight of each group. However, the increase in average heart weight of these three groups was very small. The degree of genetic determination from the cross analysis was estimated to be 45-65 percent. These results indicate that heart weight is a highly heritable trait, and that the effect of genetic factors on cardiac enlargement is larger than that of blood pressure. A similar result was cotained for the aorta weight. However, the effect of genetic factors was less important for aorta weight than for heart weight, since the degree of genetic determination was estimated to be 45-65 percent from the strain comparison and 35-60 percent from the cross analysis.

Enlargement and nucleic acid content of the cardiovascular system of several strains (SHRST/N, SHR/N, OM/N, M520/N) of rats were compared with the WKY/N strain in an atempt to characterize cardiac enlargement. Cardiac enlargement in rats can be due to either hypertrophy (increase in myocyte size), hyperplasia (increase in cell number including supporting tissue) or a combination of these factors. The sum of the indices of the degree of hypertrophy and hyperplasia calculated from the difference of heart and aorta DNA concentration and total DNA content between each strain and the WKY/N was almost equal to the degree of heart and aorta enlargement. The SHRSP/N revealed a striking hypertrophy of myocardial cells from the pre-hypertensive stage, and hyperplasia appeared gradually with the elevation of blood pressure. In contrast, the SHR/N developed a marked hyperplasia with some hypertrophy at the pre-hypertensive stage. Cardiac enlargement of the OM/N was attributed to both hypertrophy and hyperplasia. A large heart weight of the M520/N was recognized at only young age, and was due almost entirely to hyeprplasia. Aortic enlargements were related to hyperplasia. An increased RNA concentration was observed in both ventricles of the SHRSP/N, SHR/N and M520/N at four weeks of age, and all of four strains at sixteen weeks of age. A significantly higher RNA concentration was indicated in aorta of three hypertensive strains of SHRST/N, SHR/N and OM/N at established hypertensive stage. These changes might be related to manifestation of genetic or other factors such as the effect of elevated blood pressure.

Significance to Biomedical Research and Institute Programs: An understanding of the genetics of hypertension and how this genetic information is expressed is of considerable importance as we attempt to learn about the underlying factors in human essential hypertension. This is one of the major objectives of the Institute and such studies may eventually lead to an approach to predict and prevent high blood pressure and related diseases.

Proposed Course of Project: In the coming year we will establish a cell culture system to study factors which regulate protein synthesis in endothelial cells. Such cells will be prepared from both normotensive and genetically hypertensive rats.

Publications:

Tanase, H., Yamori, Y., Hansen, C.T. and Lovenberg W.: Heart Size in Inbred Strains of Rats. I. Genetic Determination of the Development of Cardiovascular Enlargement in Rats. Hypertension, 1982, (In Press).

Tanase, H., Yamori, Y., Hansen, C.T. and Lovenberg W.: Heart Size in Inbred Strains of Rats. II. Cardiovascular DNA and RNA Contents During the Development of Cardiac Enlargement in Rats. Hypertension, 1982, (In Press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01859-11 HE

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Characterization and Mechanism of Action of Dopamine-B-Hydroxylase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Jeffrey H. Hurst	Guest Worker	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sec. Biochem. Pharm.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1		

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Dopamine-B-hydroxylase (DBH) is a glycoprotein which catalyzes the conversion of dopamine to norepinephrine in sympathoadrenal cells. During the exocytotic release of norepinephrine, DBH protein is released and traverses via lymphatics into the circulatory pool where it maintains its enzymatic activity. In man and rat, serum DBH levels reflect poorly the activity of the peripheral sympathetic nervous system, suggesting the circulatory disposal rate for DBH, in addition to its entry rate, may be an important regulatory factor. Elucidation of the peripheral catabolic pathway for DBH would provide a firm foundation for understanding the regulation of DBH in the circulation.

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Objectives: The main objective of this project is to define the role of the carbohydrate compound of DBH in regulating the catabolism of DBH molecules in the circulating pool, and to define the catabolic pathway for circulating DBH.

Methods: Pheochromocytoma tumors are maintained by serial passage in the New England Deaconess Hospital strain of rats Tumors are harvested and the DBH purified according to Fong et al. (J. Neurochem 34:346-350, 1980). Homogeneity of the purified enzyme is assessed by polyacrylamide gel electrophoresis. Carbohydrate groups are cleaved enzymatically from the purified proteins using specific glycosidases such as neuraminidase, B-galactosidase, etc. Both enzymatic activity and immunological reactivity (using homologous antiserium) will be determined following glycosidase treatment.

Proposed Experiments: The relative disposal rate of native, asialo- and asialoagalacto- rat pheochromocytoma DBH will be assessed using a bolus infusion paradigm (Hurst et al; JPET 220:108-112, 1982). Should asialoDBH show a markedly faster disposal rate, we will attempt to block its catabolism by simultaneous administration of asiolofetuin. Previous work demonstrated that a constant infusion of asiolofetuin into rats would raise the endogenous levels of circulating DBH suggesting that the catabolism of the serum enzyme was being diminished (Stolk, Hurst, and Nisula; Behav. Genetics 12:37-52, 1982). Pharmacokinetic studies with radiolabelled DBH will be carried out to define the cellular site of catabolism; potential sites include hepatocytes (via the galactose-specific pathway elucidated by Ashwell and Morell [Adv. Enzymol. 41:99-128, 1974], reticuloendothelial cells (perhaps via the mannose-specific pathway reported by Kawasaki et al. [Biochem. Biophys. Res. Comm. 81:1018-1024, 1978], or spleen.

Major Findings: No work has been done on this project in the current year.

Significance to Biomedical Research and Institute Programs: DBH is the final biosynthetic enzyme in the synthesis of norepinephrine. Further, it is the only catecholamine biosynthetic enzyme which is subject to ready and repeatable measurement in man. In order to utilize effectively serum DBH measurements as a biochemical marker in clinical medicine, it will be necessary to define those factors which regulate or modulate serum DBH levels. Defining the catabolic pathway for DBH and the role of its carbohydrate moieties in this catabolism is important in understanding the regulation of DBH activity in the circulatory pool.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01865-07 HE

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Calcium-Calmodulin Dependent Activation of Tryptophan Hydroxylase by ATP and Magnesium

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Donald Kuhn	Staff Fellow	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Tryptophan hydroxylase in rat brainstem extracts is activated 2 to 2.5 fold by ATP and Mg2+ in the presence of subsaturating concentrations of the pteridine cofactor 6-methyltetrahydropterin (6MPH4). The activation by ATP-Mg2+ is also observed if the natural cofactor tetrahydrobiopterin or the synthetic cofactor dimethyltetrahydropterin is used. The activation requires ATP and Mg2+ and is not dependent on cyclic nucleotides. The ATP-Mg2+ stimulation is enhanced by calcium and can be blocked by EGTA. Removal of calmodulin from the hydroxylase containing extracts by affinity chromatography on fluphenazine-Sepharose rendered tryptophan hydroxylase unresponsive to activation by ATP-Mg2+. The readdition of calmodulin restored the ATP-Mg2+-induced activation in a calcium dependent manner. Drugs and various peptides which bind to calmodulin were found to have variable effects on the activation of tryptophan hydroxylase by phosphorylating conditions.

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PHS-6040
(Rev. 2-81)

Objectives: It has been demonstrated in our laboratory (Kuhn et al., BBRC 82: 759-766, 1978) and in a number of others that tryptophan hydroxylase, the initial and rate limiting enzyme in the biosynthesis of serotonin, can be activated *in vitro* by phosphorylating conditions (ATP-Mg^{2+}). The activation of tryptophan hydroxylase is unique in that it requires calcium and shows no apparent dependence on cyclic nucleotides. The purpose of this study was to further examine the role that calmodulin, the heat-stable calcium binding protein played in the activation of tryptophan hydroxylase by ATP-Mg^{2+} by testing the effects of various drugs and bioactive peptides which can bind to calmodulin on the activation of tryptophan hydroxylase.

Methods: Male Sprague-Dawley rats were decapitated and the mesencephalic tegmentum, which contains essentially all of the serotonin (5-HT) containing perikarya in this species, was rapidly dissected from the brain, frozen on solid CO_2 and stored in liquid N_2. Tryptophan hydroxylase was assayed by the method of Friedman et al. (J. Biol. Chem. 247:4165, 1972) as modified by Baumgarten et al. (J. Neurochem. 21:251, 1973). For phosphorylating conditions, the following substances were added to the incubation mixture (in the final concentrations indicated): ATP (0.5 mM) and Mg^{++} acetate (5 mM). The following drugs and peptides were added to control or phosphorylating reaction mixtures: chlordiazepoxide, diazepam, β-endorphin, ACTH, dynorphin, procaine, lidocaine, dibucaine, and lysine-rich histones.

Major Findings: The benzodiazepine drugs chlordiazepoxide and diazepam produced dose related inhibition of the ATP-Mg^{2+} induced activation of tryptophan hydroxylase; chlordiazepoxide was more potent than diazepam but rather high concentrations (100-200 uM) of each of these drugs were necesary for effects to be seen. Tetracaine produced an enhancement of the ATP-Mg^{2+} stimulation of hydroxylase activity whereas dibucaine produced a slight (20%) inhibition at a concentration of 2.0 mM. Both β-endorphin and dynorphin produced greater than 50% inhibition of the phosphorylation effect at concentrations of 50 M while morphine had no effect at equimolar or higher concentrations. ACTH was also an effective inhibitor of the ATP-Mg^{2+} activation and the smaller ACTH species (amino acids 1-24) was more potent that the larger ACTH species (1-38).

Finally, histone H1 was found to produce greater than 70% inhibition of the activation of tryptophan hydroxylase at very low concentrations (10 uM) and histone III-S was also quite potent, producing 60% inhibition at a concentration of 20 uM.

Significance to Biomedical Research and Institute Program: All of the drugs and peptides presently tested for antagonism of the phosphorylation-induced activation of tryptophan hydroxylase have been shown to bind to calmodulin and to inhibit various calmodulin dependent reactions. The present results establish, however, that all calmodulin binding substances do not inhibit the calmodulin dependent activation of tryptophan hydroxylase by ATP-Mg^{2+}. The most potent inhibitors are the lysine-rich histone fractions and this result may indicate that histone is simply competing with tryptophan hydroxylase for phosphorylation by a protein kinase or that histones are inhibiting the kinase. In any case, the present results indicate that certain psychoactive drugs and peptides can modulate the activation of tryptophan hydroxylase *in vitro* and these substances could also influence the *in vivo* levels of hydroxylase activity by similar mechanisms.

Proposed Course of Project: The following experiments are planned to more completely assess the role of calmodulin in tryptophan hydroxylation.

1. Purified calmodulin will be covalently bound to Sepharose-4B and this affinity matrix will be used to purify any protein kinases binding to the gel.

2. Rats will be treated chronically with a long-acting antipsychotic, fluphenazine decanoate. At weekly intervals, groups of treated subjects will be sacrificed and the brains will be assayed in the presence and absence of phosphorylating components.

3. Calmodulin will be removed from activated preparations of tryptophan hydroxylase to assess whether calmodulin plays a role in maintaining the hydroxylase in the activated state.

Publications:

1. Kuhn, D.M. and Lovenberg, W.: Calmodulin: Neurotransmitter Synthesis (Tryptophan Hydroxylase) Fed. Proc., 41:2258-2264, 1982.

2. Kuhn, D.M.: The regulation of tryptophan hydroxylase activity. In: Function and Regulation of Monoamine Enzymes: Basic and Clinical Aspects. E. Usdin, N. Weiner, M. Youdim (eds.), New York: MacMillan; 1981, In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01872-05 HE

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
CSF Levels of Pteridine Cofactor and Monoamine Metabolites Following Peripheral Administration of Tetrahydrobiopterin (Revised Title)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Leonard Miller	Guest Worker	HE NHLBI
OTHER:	Tom Insel	Clinical Associate	NIMH
	Mikka Sheinin	Visiting Fellow	NIMH
	Dennis Murphy	Chief, Clinical Neuropharm.	NIMH
	Walter Lovenberg	Chief, Sect. on Biochem. Pharm.	HE NHLBI

COOPERATING UNITS (if any)

Clinical Neuropharmacology, NIMH

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Biochemcial Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In the present investigation we administered tetrahydrobiopterin (THB) and analyzed its ability to penetrate the blood-brain barrier. THB was injected at a dose of 15-20 mg/kg into rhesus macaques kept in a restraining chair, with CSF collected by continuous sampling through cervical cannulas. THB in CSF increased in the first 90 minutes and peaked at 3-4 hours following peripheral administration. Peak levels ranged from 3-30 nmoles/ml of CSF which represents as much as a 700-fold increase over baseline levels. Within 24 hours, CSF levels of THB returned to baseline. Preliminary analysis of CSF monoamine metabolites suggests minimal changes. Further similar experiments are being performed using 6-methyl tetrahydropterin.

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PHS-6040
(Rev. 2-81)

Objectives: Degeneration of the dopaminergic nigral-striatal pathway has been one of the major neurological characteristics of Parkinson's disease. Since this important observation many years ago, clinicians have attempted to remedy this neuronal deficit by administering large doses of L-DOPA, which is subsequently converted *in vivo* to dopamine. Recently we have taken the approach that dopamine synthesis can also be affected by increasing the activity of tyrosine hydroxylase (TH), the rate limiting enzyme in dopamine synthesis. It was felt that this could be accomplished since other investigators have speculated that TH is undersaturated with respect to its cofactor tetrahydrobiopterin (BH_4). Thus administration of large doses of BH_4 if it crossed the blood-brain barrier and accumulated sufficiently in dopaminergic terminals could possibly result in increased dopamine synthesis.

Methods: Rhesus macaques were kept in restraining chairs while CSF was collected by continuous sampling through cervical cannulas, which led to a $-20^{o}C$ freezer. THB in CSF was measured by the acid and base oxidation method of Fukushima and Nixon (Anal. Biochem. 102, 176-188, 1980). The generated biopterin was prepurified by consecutive cation and anion exchange chromatography and then quantified by HPLC using a reverse phase column in combination with fluorescence detection (350/445). Monoamine metabolites were measured directly by HPLC with electrochemical detection.

Major Findings: THB in CSF increased in the first 90 minutes and peaked at 3-4 hours following peripheral administration. Peak levels ranged from 3-30 nmoles/ml of CSF, as much as a 700-fold increase over baseline levels. CSF levels of BH_4 returned to baseline after 24 hours.

CSF monoamine metabolite results suggest minimal mean changes for four animals although considerable inter-individual variation was evident. Urinary excretion of the serotonin metabolite 5-hydroxyindole acetic acid (5-HIAA) also failed to increase significantly after BH_4. Urinary excretion of cortisol showed a trend towards increasing on the active drug day. There was no evidence of behavioral change or change in heart rate following BH_4 administration.

Significance to Biomedical Research: While it appears that THB penetrates into the CSF of primates, these marked cofactor increases appear insufficient to change catecholamine or indoleamine metabolite levels. Nevertheless the inter-individual variance in the current results suggests that in certain animals or perhaps in states with deficits of cofactor, BH_4 administration might result in neurochemical and behavioral changes.

Proposed Course of Project: A similar course of experimentation is presently being pursued using 6-methyl tetrahydropterin alone and in conjunction with tyrosine.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01874-06 HE

PERIOD COVERED
October, 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Adrenergic Neurons in the Brain

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Jeffrey H. Hurst	Guest Worker	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sec. Biochem. Pharm.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8		

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Adrenaline-containing neurons in the medulla oblongata and hypothalamus have been implicated in playing a role in blood pressure regulation and hypothalamic regulatory processes. The finding by our group and others of higher levels of phenylethanolamine N-methyltransferase (PNMT) in the medullary C-1 and C-2 areas of spontaneously hypertensive rats (SHR) suggest that hyperactivity of epinephrine-synthesizing neurons may have a role in the development or maintenance of hypertension in this animal model.

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Objectives: We plan to study the properties and regulation of medullary PNMT and hypothalamic NMT activity, with a goal of understanding the role of central epinephrine neurons in blood pressure regulation.

Methods: Rat medullary PNMT and salsolinol-OMT activities will be purified using gel filtration chromatography, ion-exchange chromatography, and isoelectric focusing. Molecular weights will be assessed using commercially available protein standards on polyacrylamide gel electrophoresis. Similar procedures wil be utilized to purify and characterize the hypothalamic NMT. The N-methyltransferase activity was measured by the radiochemical assay described by Axelrod (JBC 237: 1657-1660, 1962).

Major Findings: We have found that rat medullary PMT can N-methylate phenylethanolamine, octopamine, synephrine, and metanephrine. The enzyme is inactive with phenylethylamine and tyramine. The enzyme has a pH optimum of 7.9-8.6 in Tris-HCl buffer and shows substrate inhibition of activity at high substrate levels. The specific PNMT inhibitior SKF 64139 (7,8-dichloro-1,2,3,4-tetrahydroisoquinoline) blocks the activity of medullary PNMT in a dose-dependent manner with an $IC_{50} = 10^{-8}$ M. In contrast, the NMT of rat hypothalamus shows broad substrate specificity, as it will N-methylate phenylethanolamines, phenylethylamines, N-methylphenylethanolamines, and tryptamine. The PNMT inhibitor SKF 64139 does not inhibit this enzyme, and is in fact a good substrate. This ability to N-methylate a tetrahydroisoquinoline (THIQ) represents a unique substrate for a CNSNMT, and an area of potential importance, as THIQs such as salsolinol and tetrahydropapaveroline can be formed *in vivo* via a condensation of ethanol-derived acetaldehyde and catecholamines. We have further found that salsolinol (condensation product of acetaldehyde and dopamine) is O-methylated by a medullary enzyme whose substrates, properties and pH optima suggest that it is distinct from catechol O-methyltransferase (COMT). Purified rat liver COMT is unable to use salsolinol as a substrate.

Significance of Research: The anatomical localization of PNMT-containing neurons suggests a role in blood pressure regulation and regulation/modulation of other physiological processes. By studying the properties and regulators of PNMT and related methyltransferases, we hope to gain insight into the physiological role that these enzymes play in the cental nervous system.

Proposed Course of Project: Our group and others (Turner *et al.*, Brain Res., 153:419-422, 1978), have demonstrated that medullary PNMT is subject to rapid activation in response to stress. We plan to study potential activators of purified PNMT *in vitro*, including cAMP-dependent protein kinase, calmodulin-dependent protein kinase, and sulfhydryl reagents.

Publications:

1. Yamori, Y., Horie, R., Fujiwara, M. and Lovenberg, W. Effect of Unilateral Carotid Ligation on Brainstem PNMT Activity. Eur. J. Pharmacol. 77:317-320, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01879-05 HE

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Pharmacological and Biochemical Aspects of the Functional States of Striatal Dopamine Receptors (Revised Title)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ingeborg Hanbauer	Pharmacologist	HE NHLBI
OTHER:	Maurizio Memo	Guest Scientist	HE NHLBI
	Walter Lovenberg	Chief, Sec. Biochem. Pharm.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Incubation of striatal slices for 30 min or longer in the presence of dopamine, apomorphine or SKF 38393 (all 10 uM) causes desensitization of dopamine-sensitive adenylate cyclase. The loss in responsiveness of adenylate cyclase is mediated by prolonged occupancy of D-1 recognition sites. The selective D-2 receptor agonist LY 141865 failed to elicit desensitization of dopamine-sensitive adenylate cyclase. These results were further confirmed by findings showing that haloperidol but not sulpiride blocked the desensitization elicited by dopamine. In dopamine-desensitized striatal slices the Kd for 3H-N-propylnorapomorphine binding was increased, while the activation of adenylate cyclase by NaF and cholera toxin was decreased. These results indicate that in dopamine-desensitized striatal slices the coupling of G/F protein with adenylate cyclase and dopamine recognition sites may be impaired.

PHS-6040
(Rev. 2-81)

Objectives: In the brain, various types of dopamine receptors, with diverse pharmacological and biochemical profiles, have been described. (Seeman, Pharmacol. Rev. 32:229-313, 1981). Of these, the D-1 receptor has been most characterized. In D-1 receptors the dopamine recognition site is coupled to adenylate cyclase by a GTP-binding protein (G/F). Presumably, the dopamine recognition site can occur in different conformational states, where each of these states may have a different affinity for the putative transmitter. It is unlikely that the various transitional states express different receptors, but they may merely express different functional states of the same recptor. Therefore, this project is aimed at studying dopamine receptor functioning as a supra-molecular entity which may be regulated at the level of any of its components. In order to establish the role of the transmitter recognition site, G/F protein, calmodulin, adenylate cyclase and phosphorylation of membrane proteins in dopamine receptor function we have studied the content and activity of these components in experimental conditions that cause sub- or super-sensitivity of the dopamine receptor.

Methods: Rat striatal slices were preincubated in Krebs-bicarbonate buffer pH 7.4 at constant oxygenation and thereafter incubation was continued in the presence of various dopamine receptor agonists or (and) antagonists for a prolonged period of time. At the end of the incubation, various components of the dopamine receptor complex were studied: 1) Responsiveness of adenylate cyclase to dopamine; 2) Calmodulin content in cytosol and membrane-fractions; 3) Specific ligand binding to dopamine recognition sites using ^{3}H-N-propylnorapomorphine and ^{3}H-spiroperidol as ligands; 4) Changes in G/F protein activity were studied indirectly by measuring the stimulation of adenylate cyclase by NaF and cholera toxin; 5) Phosphorylation of striatal membrane proteins.

Major Findings: Persistent occupancy of dopamine recognition sites by agonists results in a loss in the stimulation of adenylate cyclase by dopamine or other agonists with high affinity for D-1 receptors. Agonists of D-2 receptors failed to modify adenylate cyclase stimulation. Moreover, sulpiride, a D-2 receptor antagonist, failed to prevent desensitization, whereas haloperidol was able to prevent desensitization. The V_{max} of adenylate cyclase was not altered, but the ED_{50} for enzyme stimulation by dopamine was increased about 5-fold. The stimulation of adenylate cyclase in desensitized striatal slices by NaF and cholera toxin was greatly decreased. In addition, a loss in binding affinity for radiolabelled agonists in striatal membrane preparations was observed in dopamine-desensitized slices, whereas the binding affinity for radiolabelled antagonists remained unchanged. In desensitized striatal slices a decrease in calmodulin content was observed.

Significance to Biomedical Research and Institute Programs: The outcome of this research project will improve our understanding of the interaction of a neurotransmitter with its post-synaptic membrane receptor. These studies will render information on the action and on the side-effects of drugs, such as psychomimetics or neuroleptics, and will help to improve our understanding of altered receptor function in various mental disorders.

Proposed Course of Project: This project will be further extended to studies on the role of phosphorylation and dephosphorylation of membrane proteins during desensitization of striatal dopamine receptors.

Publications:

1 Memo. M., Lovenberg, W. and Hanbauer, I., Agonists-induced subsensitivity of adneylate cyclase coupled with a dopamine receptor in slices from rat corpus striatum, Proc. Natl. Acad. Sci., 79, (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01881-05 HE

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Effects of Pharmacological Manipulations on the Content of Various Neuronal Components in the Striatum.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert Levine	Pharmacologist	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.9	0.4	0.5

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Tetrahydrobiopterin (BH4) is the rate-limiting cofactor for tyrosine hydroxylase (TH) and tryptophan hydroxylase, the initial enzymes which control the synthetic rates of the catecholamines and serotonin. Prior neurochemical lesions in the nigrostriatal system of the rat with 6-hydroxydopamine (6-OHDA) indicated that the majority of BH4, TH, and GTP-cyclohydrolase (CH) are co-localized in striatal dopamine (DA) nerve terminals. Our studies using intra-striatal kainic acid (KA) injection indicated that 22 days after KA, TH and BH4 were decreased by 25% and 30%, respectively. We now demonstrate that KA causes a 30% decrease in the protein to wet weight ratio (PWWR) in the lesioned striatum, which accounts for the observed changes in TH and BH4. Thus, results which express the concentration of striatal components after KA may vary depending on whether tissue protein or wet weight is used as the denominator. Our results demonstrate that KA doesn't cause significant DA terminal damage. Also, in Parkinson's disease, the site of decarboxylation of exogenously administered L-dopa doesn't occur in neurons whose cell bodies are located in the striatum. Rather, decarboxylation probably occurs in surviving DA and possibly serotonin terminals.

Objectives: In order to more fully understand the consequences of therapeutically manipulating rates of biogenic amine synthesis by altering BH_4 levels in the central nervous system, it is important to determine the various systems in which BH_4 may have a physiological role. Through the use of specific neurochemical lesions, we have attempted to gain a further understanding of the relationship between BH_4 and central aminergic neurons as well as to investigate the likelihood of alternative roles for BH_4 in cellular function. Answers to these questions will provide useful information regarding the potential therapeutic administration of BH_4 in certain neurological and psychiatric disorders. Our studies may also unveil novel concepts regarding the nature of the interactions between BH_4 and the aromatic amino acid hydroxylase enzymes, specifically tyrosine hydroxylase.

Methods: Male Sprague-Dawley rats (250-300 g) were used for all 6-hydroxydopamine (6-OHDA) and kainic acid (KA) lesion experiments. Animals were anesthetized with Chloropent (2.2 ml/kg) and placed in a David Kopf stereotaxic instrument. In one set of animals, 6-OHDA was injected (8 µg/4 µl) in the left substantia nigra (SN) to destroy the nigrostriatal dopaminergic neurons. The coordinates from bregma used for injection of 6-OHDA in the SN were as follows: anterior-posterior (AP) = -5.2 mm, medial-lateral (ML) = +2.0 mm, dorsal-ventral (DV) = -7.1 mm (from cortical surface). Two weeks after the operation, the effectiveness of the lesion was examined by monitoring apomorphine-induced rotational behavior. After apormorphine injection, animals were placed in a round bowl and turns were monitored by an observer for thirty minutes after an initial five minute post-injection interval during which turning was not quantitated. Greater than three clockwise turns/minute for a thirty minute period constituted a successful lesion. Only positive-turning animals were used for subsequent biochemical assays described below. 6-OHDA treated animals were sacrificed two weeks after testing rotational behavior.

In a separate group of animals, KA was injected (2 µg/1 µl) unilaterally in the left striatum to destroy striatal cell bodies and interneurons, while causing minimal damage to striatal nerve terminals. The coordinates from bregma used for KA injection in the striatum were as follows: AP = 0.0 mm, ML = +3.0 mm, DV = -4.0 mm (from cortical surface). The success of KA lesions was determined by measuring substance P-like immunoreactivity (SPLI) in both the anterior striatum (substance P cell body location) and SN (SP terminal area). These structures comprise the striatonigral SP pathway which is reciprocal to the nigrostriatal dopamine pathway. KA-treated animals were initially sacrificed 22 days after the lesion. In subsequent experiments, groups of 6 rats each were sacrificed at 2, 7, 14, 22 and 30 days after intrastriatal KA injection.

Control and lesioned animals were sacrificed by decapitation, brains were quickly removed and the appropriate areas were dissected from coronal slices which were obtained by using single-edged razor blades places in a slotted plexiglass brain block which was maintained at 4°C on ice. All dissected tissues were frozen in liquid nitrogen until time of assay.

Substance P-like immunoreactivity was measured in the substantia nigra and striatum by radioimmunoassay which was previously developed in our laboratory. Reduced pterin cofactor content in certain experiments was measured in the nigra and striatum by the purified phenylalanine hydroxylase assay with minor modifications as described in detail in last year's annual report.

The more specific high pressure liquid chromatography (HPLC) assay recently described by Fukushima and Nixon (1980) was used to measure oxidized and total striatal biopterin content. This method was described in detail in last year's report. In essence, the assay consists of a differential iodine oxidation procedure which allows one to measure the various oxidative forms of biopterin.

Aromatic amino acid decarboxylase (AAAD) activity was determined by measuring the amount of ^{14}C released from ^{14}C-L-DOPA. Tyrosine hydroxylase activity was determined by the standard tritium release assay.

Major Findings: We reported last year that a 6-OHDA lesion of the SN depleted biopterin (B) content and the activity of GTP-cyclohydrolase (CH), the initial enzyme in BH_4 synthesis, by approximately 75%, whereas tyrosine hydroxylase (TH) was diminished by 90%. These results suggested that the majority of B and its biosynthetic system are highly co-localized in the dopamine neurons of the nigrostriatal system. The difference between the degree of depletion of TH (93%) versus B and CH (both 70%) probably represents the cofactor synthetic system which is required for the activity of tryptophan hydroxylase and contained within the serotonergic nerve terminals innervating this region. Aromatic amino acid decarboxylase (AAAD) catalyzes the conversion of L-dopa to dopamine. It has been reported that unilateral 6-OHDA injection in the nigra causes an 80% reduction in AAAD activity in the ipsilateral striatum when TH activity is reduced by greater than 90%. This would indicate that the majority of striatal AAAD is also contained within the dopamine terminals while the AAAD remaining after the lesion is located elsewhere. AAAD is also required for serotonin synthesis, thus some portion of the remaining AAAD in the striatum after the 6-OHDA lesion probably resides in the serotonin terminals which are not destroyed by 6-OHDA. It has also been demonstrated that a small percentage of AAAD is associated with blood vessels. However, the existence of another neuronal compartment in striatum which contains AAAD and is not associated with dopamine or serotonin terminals or blood vessels has been suggested by other workers. In their experiments, intrastriatal KA injection caused a 20% decrease in striatal AAAD activity while there was no apparent change in dopamine content or TH activity 14 days after the lesion (all data was expressed as per milligram of tissue wet weight). Intrastriatal KA injection is purported to destroy striatal cell bodies and interneurons while not damaging nerve terminals or axons of passage through the striatum. Since TH activity and dopamine content were equivalent to control in their experiments, they reasoned that KA caused no dopamine nerve terminal damage. However, since KA significantly reduced striatal AAAD activity it was surmised that another neuronal compartment existed in the striatum which contained a significant portion of striatal AAAD. Thus, other workers propose that this pool of AAAD may be responsible for the decarboxylation of exogenously administered L-DOPA to patients with Parkinson's disease. In our experiments, we examined the effects of unilateral intrastriatal KA injection on various neuronal contents in the striatum. Contrary to their hypothesis, our data do not support the existence of a KA-sensitive neuronal system containing AAAD in striatum. Furthermore, it appears that the denominator (protein vs wet weight) used to express results from striatal KA lesion experiments is crucial in order to avoid drawing erroneous conclusions concerning functional and neuroanatomical relationships in the striatum.

Our initial experiments with KA indicated that TH activity and BH_4 content in striatum 22 days after KA were decreased by 25% and 30%, respectively. Since TH and BH_4 are co-localized in striatal dopamine terminals, these results

suggested that KA might cause terminal damage which could only be detected biochemically by a decrease in TH activity at 22 days after the lesion. Since it had been reported that AAAD activity (expressed as a function of tissue wet weight) was also decreased after KA, we investigated the time course from 2 to 30 days of KA effects on striatal TH and AAAD activities, and BH_4 content. Our initial experiments also indicated that the relationahip between tissue protein content and wet weight of the injected striatum was altered by KA when compared to control striata. Therefore, the striatal protein to wet weight ratio (pwwr) was also monitored in control and lesioned striata. We also examined the effects of unilateral 6-OHDA injection in the nigra on striatal pwwr to ascertain whether this lesion, which also alters the content of striatal neuronal components, could influence striatal pwwr. Intranigral 6-OHDA injection, which successfully eliminates the majority of NS DA neurons, had no effect on striatal pwwr. In contrast, intrastriatal KA injection caused a 30% decrease in striatal pwwr which persisted up to one month after the lesion. This alteration in pwwr by KA has a profound influence on the content of various striatal components depending on whether tissue protein or wet weight is used as the denominator for expressing results. The net effect of a decrease in striatal pwwr after KA appears to be a dilution of all surviving elements in the striatum. The reduction in pwwr is maximal at 2 days and remains significantly reduced up to 30 days. When TH, AAAD, and BH_4 are expressed on a tissue wet weight basis, all are significantly reduced on the lesioned side. However, when the data are expressed on a mg protein basis, the results exhibit a markedly different profile. Striatal TH activity is elevated at 2 and 7 days to 20% and 35% and its activity is equivalent to control at 14, 22, and 30 days. Striatal AAAD is elevated 10% at 2 days and is equivalent to control at 7, 14, and 30 days, while demonstrating a drop of 20% at 22 days. Biopterin content in the striatum is essentially unaltered by KA when results are expressed as a function of tissue protein. It cannot be determined from these studies the mechanism by which KA decreases the pwwr in the striatum. A theoretical consideration of these results would indicate that the most likely effect of intrastriatal KA injection is to cause an increase in total striatal weight as a result of tissue swelling with fluid while not changing significantly the total amount of striatal protein. Regardless, the net effect of a decrease in striatal pwwr after KA appears to be a dilution of all surviving elements in the striatum. Therefore, it appears that the most realistic method for expressing results generated from measurements on KA-lesioned tissue is on a tissue protein basis.

The site of decarboxylation of exogenously administered L-DOPA to patients with Parkinson's disease has been the subject of intensive investigation. It is classically thought that decarboxylation occurs in surviving DA terminals. It has also been suggested that serotonin terminals in the striatum might contribute to the decarboxylation of L-DOPA in Parkinson's disease. The recent proposal by Wurtman and coworkers of an alternative KA-sensitive neuronal compartment containing AAAD in striatum provides yet another possible source of decarboxylation of exogenously administered L-DOPA. Contrary to their results, our findings do not support the concept that AAAD exists in a KA-sensitive neuronal compartment in the striatum. Since it appears that KA-sensitive cells in striatum do not contain AAAD, it is likely that in Parkinson's disease, the decarboxylation of exogenously administered L-DOPA occurs in surviving dopamine and possibly serotonin nerve terminals.

Significance to Biomedical Research and Institute Programs: Our recent results have clearly dispelled the notion that a KA-sensitive neuronal pool containing

decarboxylase exists in the striatum. The alteration of the striatal protein to wet weight ratio by KA is an important observation which will be of interest to the many researchers who work with KA. This effect must be taken into account in order to insure the proper evaluation and interpretation of results. This may prevent the development of erroneous hypotheses from data obtained by the local injection of KA. These studies will be of interest to neurologists since the site of exogenously administered L-DOPA in Parkinson's disease has been intensively studied. Based on our results, the most likely location for decarboxylation of exogenously administered L-DOPA is within DA and possibly serotonin nerve terminals and not within neuronal cell bodies in striatum.

Proposed Course of Project: Since BH_4 plays such a pivotal role in biogenic amine synthesis and there are indications that nigrostriatal dopamine neurons are involved in the symptomology associated with Parkinson's disease, our studies will continue to examine the effects of various pharmacological manipulations on the nigrostriatal system.

To provide information which may be necessary for the collaborative clinical study, the time course of BH_4 and synthetic analogues entry into the CNS will be determined after I.P. or I.V. administration. To determine if BH_4 levels are capable of elevating dopamine synthesis in the striatum, BH_4 and synthetic analogues will be administered and the major metabolites of dopamine (DOPAC and HVA) will be monitored as an index of turnover of this neurotransmitter.

We will also investigate the effects of various psychoactive drugs on the content of striatal BH_4 in an attempt to see if the behavioral effects of these agents are acting through an effect on the cofactor system. This investigation should lead to a more fundamental understanding of the relationship between BH_4 and the hydroxylase enzymes.

Publications:

Levine, R.A., Miller, L.P., and Lovenberg, W.: Tetrahydrobiopterin in Striatum: Localization in Dopamine Nerve Terminals and Role in Catecholamine Synthesis. Science 214: 919-921, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01884-04 HE

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Enkephalin-like Peptides and Cardiovascular Control

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Hypertension-Endocrine Branch

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Secretion of enkephalin-like peptides from adrenal glands into the circulation was studied in dogs with indwelling cannulae in the lumbar adrenal vein. Splanchnic nerve stimulation causes a voltage-dependent increase of met-enkephalin immunoreactive peptides and catecholamines in adrenal venous plasma. The effect of splanchnic nerve stimulation was mimicked by dimethylphenylpiperazinium and was blocked by hexamethonium. These results indicate that, similar to catecholamines, the release of met-enkephalin-like peptides from adrenal chromaffin cells is mediated through nicotinic receptors. Treatment of dogs with reserpine, unlike for catecholamines, did not impair storage of met-enkephalin-like peptides in adrenal chromaffin cells. Splanchnic nerve stimulation released the peptides into the adrenal vein at a similar rate as in saline-treated dogs. In reserpine-treated dogs splanchnic nerve stimulation caused a drop in arterial blood pressure, which was reversed by naloxone.

561

PHS-6040
(Rev. 2-81)

Objectives: In adrenal chromaffin cells various molecular forms of polypeptides that cross-react with met-enkephalin-antisera are stored in granules together with catecholamines and soluble proteins. On the other hand, in splanchnic nerve terminals these neuropeptides were shown to coexist with acetylcholine. The simultaneous presence of recognition sites for acetylcholine and met-enkephalin-like peptides on chromaffin cell membranes suggests that these peptides may function as cotransmitters and regulate the responses to cholinergic receptor stimulation. The scope of this project is to study 1) the synaptic mechanisms that regulate the release of catecholamines and met-enkephalin-like peptides from adrenal chromaffin cells, 2) to characterize the peptides released into the adrenal lumbar vein and 3) to obtain information on the physiological action of these peptides after being released from adrenal medulla.

Methods: Collection of adrenal venous blood: Female American foxhounds (20-25 kg.) anesthetized with pentobarbital were fitted with an indwelling catheter in the left lumbar adrenal vein. The sampling of adrenal venous blood was carried out in anesthetized dogs. In some animals the splanchnic nerve was transected and the stump leading to the adrenal gland was electrically stimulated at constant voltage (10 V) and varying frequencies (1-9 Hz). The mean arterial blood pressure was recorded before and during electical stimulation of the splanchnic nerve.

Measurements of met-enkephalin-like peptides in adrenal venous plasma: The procedure used is suitable for separation and characterization of met-enkephalin-like peptides of molecular weight smaller than 1800. Plasma was diluted with equal volumes of 0.5 N CH_3COOH and heated at 90°C for 15 min. After centrifugation at 8000 x g the supernatant fraction was passed over a SEP-PAK C_{18} column (Waters, Millipore) or a Biogel P_2 column. The respective eluates were further chromatographed on HPLC (Waters) and the met-enkephalin, leu-enkephalin and met-enkephalin-pro-phe were assayed radioimmunochemically.

Major Findings: In anesthetized dogs electrical stimulation of the splanchnic nerve at constant voltage (10 V) with increasing frequency from 1 to 9 Hz elicits a gradual increase in the amount of met-enkephalin, leu-enkephalin, norepinephrine and epinephrine released into the adrenal lumbar vein. The effect of splanchnic nerve stimulation on plasma levels of met-enkephalin-like peptides was antagonized by hexamethonium but not by atropine. These results suggest that the release of met-enkephalin-like peptides is regulated by nicotinic receptors in a similar fashion as described for catecholamines. In dogs which were injected with reserpine to deplete the adrenal catecholamine stores, splanchnic nerve stimulation caused a release of met-enkephalin-like peptides similar to that in saline-treated dogs. These results indicate that although met-enkephalin-like peptides and catecholamines are stored in the same vesicles, reserpine specifically impairs the storage of catecholamines but not that of the peptides. In addition, in reserpinized dogs splanchnic nerve stimulation elicits a fall of the basal systolic and diastolic blood pressure. Injection of naloxone at the nadir of hypotension reversed the decrease in blood pressure elicited by splanchnic nerve stimulation. This finding suggests that met-enkephalin-like peptides released from adrenal medulla may act on enkephalin receptors in vascular smooth muscle.

Significance to Biomedical Research and Institute Programs: Enkephalin-like peptides have been studied more extensively on their control of pain threshold, but the physiological function of these peptides does not appear to be limited to this effect. Since narcotic agents of clinical values are always showing respiratory and vasodepressant effects and since the injection of morphine increases the

content of (met^5)-enkephalin-like peptides in venous blood it appears important to reveal the mechanism of action of these peptides on the cardiorespiratory system.

Proposed Course of Project: Future experiments are planned to study the functional role of met-enkephalin-like peptides present in splanchnic nerve terminals. Studies are planned to determine whether occupancy of enkephalin receptors located on adrenal chromaffin cells regulate the release of met-enkephalin-like peptides and catecholamines from their storage sites.

Publications:

1. Costa, E., Guidotti, A., Hanbauer, I., Hexum, T.D., Saiani, L. and Yang, H.-Y.T.: Regulation of cholinergic transmission in adrenal medulla, In: Cholinergic Mechanisms, (eds., G.C. Pepeu and H. Ladinsky), Adv. Behavioral Biology, Vol. 25, p. 143-154, Plenum Press, New York, 1981.

2. Govoni, S., Hanbauer, I., Hexum, T.D., Yang, H.-Y.T., Kelly, G.D. and Costa, E.: In vivo characterization of the mechanisms that secrete enkephalin-like peptides stored in dog adrenal medulla, Neuropharmacology, 20, 639-645, 1981.

3. Costa, E., Guidotti, A., Hanbauer, I., Hexum, T.D., Saiani, L., Stine, S. and Yang, H.-Y.T., Regulation of acetylcholine receptors by endogenous cotransmitters: studies of adrenal medulla, Fed. Proc. 40, 160-265, 1981.

4. Hanbauer, I., Govoni, S., Majane, E.A., Yang, H.-Y.T. and Costa, E., In Vivo regulation of the release of met-enkephalin-like peptides from dog adrenal medulla, In: Regulatory peptides: Functional and Pharmacological aspects (ed. M. Trabucchi), Raven Press, New York, (in press).

5. Hanbauer, I., Kelly, G.D., Saiani, L. and Yang, H.-Y.T., [Met^5]-enkephalin-like peptides of the adrenal medulla: Release by nerve stimulation and functional implications. Peptides (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01885-04 HE

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

The action of Substance P on the working rat heart (Revised Title)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Hypertension-Endocrine

SECTION
Biochemical Pharmacology

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The effect of substance P on the cardiodynamics of an isolated, working rat heart preparation was examined. The peptide was found to have no influence on aortic pressure, cardiac output, or cardiac work. However, a 10-15% reduction in coronary flow was observed over the concentration range of 10^{-8} to 10^{-6}M. The octapeptide analogue of substance P also exhibited a similar vasoconstrictive effect, but at a much lower concentration; its ED_{50} of 2×10^{-13}M was several orders of magnitude less than the ED_{50} of substance P ($ED_{50} = 3.5 \times 10^{-8}$M). Perfusion with buffer containing the peptidase inhibitor bacitracin (10^{-4}M) eliminated the vasoconstrictive effect of substance P but not that of the octapeptide substance P. Thus, we conclude that a breakdown product (e.g. the octapeptide) of Substance P is responsible for the observed decrease in coronary flow.

564

PHS-6040
(Rev. 2-81)

Objectives: Substance P has been shown to be present in the coronary vasculature of the heart. Therefore we decided to examine the cardiac effects of Substance P. We were also interested in determining if Substance P is the active peptide or if these actions could be produced by a peptide fragment of Substance P.

Methods: Isolated hearts were perfused on the working heart model within 1 min following removal from the animal. The perfusion apparatus consisted of two completely separate non-recirculating systems with a minimum of dead space in switching between the two buffer systems. The aorta was cannulated and the heart was perfused with buffer from a reservoir placed 100 cm above the heart. Similarly, the left atrium was attached to a cannula and received buffer from a filling reservoir maintained at a pressure of 13 cm H_2O. The hearts were paced at 300 beats per minute by means of a Grass. F.D. 9 stimulator. Coronary flow was determined by severing the right pulmonary artery, the coronary effluent emptying into the right side of the heart was pumped out and collected. Aortic pressure was determined by use of Statham P23Gb pressure transduced placed in a side arm directly above the aorta and results were recorded on a Gould 2-channel Brush 220 recorder Cardiac work (pressure volume work) was calculated according to the equation: C.W. = total cardiac output + mean aortic pressure x 10^{-5}.

The standard perfusate was Krebs-Henseleit bicarbonate buffer supplemented with 5 mM glucose, and 2.5 units/l insulin. The buffer was warmed to 30`C and aerated with a 95% O_2 - 5% CO_2 mixture to maintain pH at 7.4. Hearts were allowed to stabilize for 15 min in normal buffer prior to switching to buffer supplemented with the peptide used.

Major Findings: Cardiac perfusion in the presence of substance P is shown in Table I. From this data, 1 x 10^{-6}M substance P has no influence on aortic pressure, cardiac work, or cardiac output in the rat heart. However, SP did induce a reduction in coronary flow which was dose dependent (Fig. 1). The reduction in coronary flow was observed within 15 seconds after switching from control buffer to buffer containing substance P. The threshold response was observed at 1 x 10^{-8}M and a maximum decrease in coronary flow of approximately 12% was observed at 1 x 10^{-6}M. The IC_{50} for reduction in coronary flow, calculated from the log of the data points was 3.5 x 10^{-8}M.

Octapeptide substance P was also examined for its effects on the perfused rat heart (Table I). Similar to substance P, the octapeptide substance P had no effect on cardiac function but did produce a similar 12% reduction in coronary flow similar to SP (Figure 1). However, the concentration of SP_{4-11} necessary to elicit this response was considerably lower than for SP. The IC_{50} for octapeptide substance P-induced coronary arterial constriction was 2 x 10^{-13}M and the maximal effect was observed at 1 x 10^{-12}M.

Since the octapeptide, at a much lower concentration, produces a similar reduction in coronary flow to that of substance P, it is conceivable that substance P may be subject to membrane associated protease modificaton to produce the more active SP_{4-11}. Therefore, we tested the effect of the non-specific protease inhibitor, bacitracin, in blocking the coronary vasoconstrictor action of substance P (Table II). Perfusion of the isolated rat heart with 1 x 10^{-4}M bacitracin failed to show any cardiac effects. However, this concentration of bacitracin abolished the reduction in coronary flow produced by 1 x 10^{-6}M SP. In contrast, the reduction in coronary flow observed with 1 x 10^{-11}M SP_{4-11} was unaffected by the presence of 1 x 10^{-4}M bacteracin in the perfusion media. Thus, inhibition of

cardiac membrane proteases by bacitracin can abolish the coronary vasoconstrictive effect of SP, but has no effect on the action of octapeptide Substance P.

Significance to Biomedical Research and Institute Programs: In the working rat heart model, perfusion with substance P was shown to reduce coronary flow. However the octapeptide analogue was more potent than substance P. The fact that bacitracin, a protease inhibitor, was able to block the reduction in coronary flow of only substance P suggests that a vascular protease may convert substance P to the very potent octapeptide.

Proposed Course of Project:

1. Examine the effects of various other substance P fragments in altering coronary flow.

2. Examine the effects of various protease inhibitors to attempt to characterize the specificity of the protease.

3. In conjunction with the Cardiology section, we will examine the amount of substance P immunoreactivity in plasma from the coronary sinus following cardiac catherization.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 03501-04 HE

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Effects of Morphine on Calcium-Regulated Phosphorylation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The administration of morphine in vivo (3-30 mgµkg) produced a dose-dependent increase in the calcium-dependent phosphorylation of three synaptosomal cytosolic proteins having molecular weights of 50,000, 55,000 and 60,000. This effect, which could be abolished by pre-treatment with naloxone, was also observed following the administration of levorphanol and l-methadone. The analgesically inactive stereoisomers, dextrorphan and d-methadone, were ineffective. In rats treated chronically with morphine, phosphorylation patterns were similar to those observed in placebo-treated controls. Morphine was also less effective in stimulating phosphorylation when administered acutely as a challenge dose to chronically-treated animals.

567

PHS-6040
(Rev. 2-81)

Objectives: A number of recent studies have demonstrated neurochemical changes in the central nervous system following acute and chronic administration of morphine. In particular, morphine has been reported to alter the activity of adenylate cyclase, as well as the levels of cAMP, in several brain regions. Morphine also alters both the levels and binding of calcium in brain. Since both cAMP and calcium are known to stimulate protein phosphorylation in synaptic membranes and synaptosomal cytosol, morphine administration might also influence protein phosphorylating systems in brain. Such systems, which include the enzymes protein kinase and phosphoprotein phosphatase, are probable links in the series of biochemical signals linking receptor stimulation and physiological effects, and thus represent a locus at which the effects of endogenous and exogenous opiates might be mediated. The objective of the present investigation was therefore to examine the effects of acute and chronic administration of morphine and related drugs on calcium and cAMP-dependent phosphorylation of cytosolic proteins from rat striatal synaptosomes.

Methods: Crude synaptosomal lysate was prepared from a striatal homogenate by differential centrifugation. The resultant supernatant fraction was used as the source of protein kinase. The net incorporation of phosphate into striatal synaptosomal cytosolic proteins was assayed by standard procedures as described in previous years.

Proteins were resolved on SDS polyacrylamide slab gels, and autoradiography was carried out for a period of 1-2 days using Kodak RP x-ray film. The incorporation of phosphate into specific protein bands was determined by microdensitometry of the autoradiographs.

Major findings: As previously reported, in vivo administration of morphine results in a maximal increase of 200% in calcium-dependent phosphorylation of synaptosomal cytosolic proteins with molecular weights of 50,000, 55,000 and 60,000. Similar effects were observed following levorphanol or ℓ-methadone treatment, but not after dextrorphan or d-methadone. The effect of morphine was blocked by pretreatment with naloxone. Phosphorylation patterns observed in chronically treated rats were similar to those observed in placebo-implanted controls, implying that tolerance to this neurochemical effect of morphine develops within 72 hours of continuous exposure to morphine.

Significance to Biomedical Research and Institute Program: Although an involvement of opiate action in the dynamics of several neurotransmitter systems has been demonstrated, little progress has been made toward a delineation of the specific molecular mechanism(s) involved in the acute and chronic effects of narcotic analgesics.

The studies described above demonstrate effects of acutely administered morphine on a calcium-regulated phosphorylation system in synaptosomal cytosol, and further reveal that tolerance to these effects occur if animals are chronically treated with morphine. Thus, calcium-regulated protein phosphorylation represents one possible locus of opiate action that may account for some of the acute and chronic effects of these drugs. Furthermore, calcium sensitive phosphorylation systems may be of physiological as well as pharmacological importance since the effects of the endogenous opiates, the endorphins, may also be mediated through an effect on calcium-regulated protein phosphorylation.

Proposed Course of Project: The effects of morphine on protein phosphorylation may well be mediated by changes in neuronal Ca^{2+} levels or binding. However, calmodulin distribution in striatal tissue slices is altered by incubation of the slices in the presence of morphine. This implies that the effect of morphine on protein phosphorylation could be mediated via increases in calmodulin levels. Therefore, future experiments will be designed to examine the effect of acute and chronic morphine treatment on calmodulin levels in synaptic plasma membranes and synaptosomal cytosol, as well as on the subcellular distribution of calmodulin. Changes in these parameters will be related to changes in protein phosphorylation patterns.

In addition, the effects of morphine on other neurochemical indices of receptor stimulation, such as adenylate cyclase activity and receptor sensitivity, will be studied in an attempt to delineate the series of biochemical events which mediate the observed effects on phosphorylation.

The analgesic action of morphine and its effect on Ca^{2+}-regulated phosphorylation do not exhibit similar time course characteristics. Inasmuch as the striatum does not seem to be a major site for the mediation of the analgesic effects of opiates, it is likely that the effects observed in the present study are linked to actions other than analgesia. Thus, other brain areas containing neuroanatomical sites linked to specific effects of opiates, including analgesia, will be examined for effects on phosphorylation similar to those reported for rat striatum.

Publications:

O'Callaghan, J.P., Juskevich, J.C. and Lovenberg, W. (1982) The effects of morphine on calcium-regulated phosphorylation of synaptosomal cytosolic proteins from rat striatum, J. Pharmacol. Exp. Ther. 220:696-702.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03503-04 HE

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Characterization of Proenzyme I from Rat Brain

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Vivian S. Zabrenetzky	NIH Postdoctoral Fellow	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.0	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A trypsin-sensitive, cyclic nucleotide independent prokinase from rat brain supernatant contains a calmodulin-like subunit. It phosphorylates histone type II in a Ca2+-dependent manner, and histone type H-1 in a phospholipid dependent manner. It is inhibited by fluphenazine and fluphenazine-Sepharose affinity chromatography can dissociate its calmodulin subunit.

HPLC and sedimentation equilibrium show that the enzyme contains several subunits without the presence of contaminating proteins.

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Objectives: To determine whether calmodulin is a subunit of the cyclic nucleotide independent prokinase and whether the preparation is pure.

Methods: The enzyme was purified by DEAE, and Sephadex G-100 chromatography and isoelectrofocusing. Polyacrylamide slab gel electrophoresis (PAGE) was used to determine the subunit structure of the enzyme. Fluphenazine and fluphenazine-Sepharose affinity chromatography were used to separate the calmodulin subunit from the enzyme. HPLC and sedimentation equilibrium were used to determine whether the enzyme was pure.

Major Findings: Highly purified prokinase C was obtained by the procedure described above. Fluphenazine could block Ca^{2+} stimulation of kinase C activity toward histone type II. When fluphenazine is linked to the affinity matrix Sepharose 4 B, any calmodulin containing moiety will be linked to the matrix in the presence of Ca^{2+} and released by EGTA. When a dialysate of prokinase C was put over such a column, its activity was dissociated into a trypsin activated fraction (Ca^{2+} wash) and a trypsin inactivated fraction (EGTA wash). The addition of Ca^{2+} to the reaction mixture caused enhanced activity in EGTA wash (+) trypsin such that levels were equal to those of (-) trypsin.

Analysis of the column fractions by SDS-PAGE revealed the presence of a low molecular weight band that co-migrates with calmodulin. Several additional bands appeared in the 50,000 molecular weight range.

Analysis of the prokinase by high performance liquid chromatography on a Toyosota column showed that the enzyme has subunit structure. These results were confirmed by sedimentation equilibrium studies on a Beckman airfuge. When different enzyme preparations were run at different speeds, a linear funtion of activity vs distance from the roter was observed. This is indicative of a singular molecular species of varying subunit sturcture.

Significance to Biomedical Research and Institute Programs: Prokinase C contains calmodulin as a subunit and is therefore only one of three kinases (myosin light chain kinase and phosphorylase kinase) to have such a subunit. It has long been established that calcium modulates neuronal transmission and that phosphorylation mediates this process. Since the prokinase contains calmodulin it could be a link between the two processes. It may provide this link by activating tryptophan hydroxylase thereby regulating serotonin synthesis in a calcium dependent manner. Or, it may phosphorylate synaptic membrane proteins to maintain the proper homeostatic level of membrane phosphorylation in preparation for synaptic transmission.

This enzyme can phosphorylate myosin light chain and myosin light chain kinase. This may be an important mechanism for activation of myosin light chain kinase.

Proposed Course of Project: The primary goal of identifying calmodulin as a subunit of the enzyme, has been achieved. Additional studies, by other investigators, concerning the role of the enzyme in tryptophan hydroxylase and myosin light chain kinase regulation could be continues.

Publications:

1. Zabrenetzky, V., Bruskwick, E. and Lovenberg, W.: Calcium stimulation of protein kinase C in the absence of added phospholipids. (1981) Biochem. Biophys. Res. Comm. 102:135-141.

571

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03505-04 HE

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Effects of Pteridine Cofactors on the Stability of Tryptophan Hydroxylase and Tryosine Hydroxylase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Donald M. Kuhn	Staff Fellow	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI
	Belle Ruskin	Chemist	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The stability of tryptophan and tyrosine hydroxylases was studied in vitro. The activity of tryptophan hydroxylase decays rapidly when preincubated at 37 C for up to 60 min. The inclusion of tetrahydrobiopterin (BH4), 6-MPH4, or dimethyl-tetrahydropterin (DMPH4) in the preincubation mixture significantly protects tryptophan hydroxylase from 02-thermal inactivation. The protective effects were provided only by reduced cofactor since biopterin did not prevent inactivation. Tyrosine hydroxylase, on the other hand, is stable throughout a 60 min preincubation and the inclusion of BH4, 6MPH4, or DMPH4 in the preincubation causes a loss of catalytic activity. The inhibitory substance is not a peroxide or superoxide anion, nor is it the cofactor or catecholamine end-product. It appears that exposure of tyrosine hydroxylase to its cofactor BH4 in the absence of the substrate tyrosine leads to the loss of activity.

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PHS-6040
(Rev. 2-81)

Objectives: Tryptophan and tyrosine hydroxylase are the initial and rate limiting enzymes in the synthesis of serotonin and catecholamines, respectively. Purification and characterization of these important enzymes has been hindered as a result of their instability, especially tryptophan hydroxylase (Kuhn et al., JBC 255: 4137-4143, 1980). Since it is a general phenomenon that many enzymes are more stable in vitro in the presence than in the absence of their substrates, we investigated whether the pteridine cofactors (cosubstrates) would alter the stability of either tryptophan or tyrosine hydroxylase.

Methods: Tryptophan hydroxylase was assayed in rat mesencephallic tegmental extracts by the method of Baumgarten et al (J. Neurochem. 21:251, 1973). Tyrosine hydroxylase was assayed in rat striatal extracts by the method of Lerner et al. (Neurochem. Res. 3:641, 1978). For stability studies, enzyme-containing extracts were incubated at 37°C in room air for 0-60 min in the presence or absence of BH_4, $6MPH_4$, or $DMPH_4$. Aliquots were removed at 15 min intervals for the appropriate enzyme assay.

In some experiments, preincubated striatal extracts were chromatographed on Dowex 50 or were dialyzed against homogenization buffer to remove small molecules.

Major Findings: Preincubation of tryptophan hydroxylase at 37°C for varying periods of time leads to a loss of catalytic activity. After 30 min of preincubation, activity is reduced to approximately 50% of starting activity. The addition of BH_4 (0.25 mM), $6MPH_4$ (1.0 mM), or $DMPH_4$ (0.5 mM) to the preincubation mixture protects tryptophan hydroxylase from inactivation. To exert protective effects the pteridine cofactors must be chemically reduced since the oxidized form of BH_4, biopterin, is without effect. Tyrosine hydroxylase, on the other hand, is quite stable throughout the entire preincubation period. After 60 min preincubation there is not more than a 10% loss of catalytic activity. The addition of BH_4, 6 MPH_4, or $DMPH_4$ surprisingly caused a rapid loss of tyrosine hydroxylase activity. As before, the inhibitory effects of the pteridines was dependent on the reduced forms since neither biopterin nor dihydrobiopterin caused a loss of activity.

It is known that catecholamines exert end-product inhibition on tyrosine hydroxylase and it is possible that some catecholamines were synthesized during preincubation. To test this possibility, extracts were chromatographed on Dowex 50 resin to remove catecholamines after preincubation. This ion-exchange step partially reversed the BH_4-induced inhibition. The exogenous addition of dopamine produced profound inhibition of tyrosine hydroxylase which was completely reversed by Dowex chromatography, ruling out an effect of dopamine. Furthermore, dialysis of BH_4-inhibited tyrosine hydroxylase returned activity to control levels. Incubation of striatal extracts (in the presence of BH_4) with catalase, peroxidase, or superoxide dismutase did not prevent the loss of activity, excluding peroxides or superoxide radicals as the inhibitors. Finally, since BH_4 could serve as a reducing agent itself, dithiothreitol or ascorbic acid were incubated with tyrosine hydroxylase and these agents had no effect on the activity of the hydroxylase nor did they alter the BH_4 induced inhibition.

Finally, the pH dependency of the inhibitory effects of BH_4 were investigated. The usual effect of BH_4 at pH 6.0 varied depending on the particular buffer used (e.g., more inhibition with Mes than with sodium phosphate at pH 6.0) and at higher pH (7.5) the inhibitory effects of BH_4 were largely prevented.

Significance to Biomedical Research and Institute Program: These experiments indicate that the pteridine cosubstrates can stabilize tryptophan hydroxylase and the instability of this enzyme during purification may result from separation of the endogenous BH_4 from the hydroxylase. With this information, the use of the pteridines as ligands for affinity chromatography could enhance the purification of tryptophan hydroxylase because of the speed and specificity with which this process can be accomplished and it should also help stabilize the enzyme during purification. The pteridine-induced inhibition of tyrosine hydroxylase is surprising and these results indicate that, under the appropriate conditions, BH_4 can cause destabilization of the enzyme. Taken together, these data suggest that BH_4 binds to tyrosine hydroxylase and, in the absence of tyrosine, produces a reduction in catalytic activity.

Proposed Course of Project

1. Continue studies on enzyme stabilization and purification using affinity chromatography.

2. Investigate the interaction of oxygen and tyrosine on BH_4-inhibition.

Publications:

Kuhn, D.M. and Lovenberg, W.: Structure and function of hydroxylase enzymes. In: Handbook of Neurochemistry, Vol. 4, A. Lajtha (ed.), 1981, in press.

Kuhn, D.M. and Lovenberg, W.: Serotonin and metabolites. In: Methods in Biogenic Amine Research, I. Najatsu and T. Nagatsu (eds.), 1981, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03510-03 HE

PERIOD COVERED
October 1, 1981 to September 31, 1982

TITLE OF PROJECT (80 characters or less)

Calcium-regulated Protein Phosphorylation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Judith Juskevich	Staff Fellow	HE NHLBI
Other:	Donald Kuhn	Senior Staff Fellow	HE NHLBI
	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We previously described a calcium- and calmodulin (CaM)-dependent protein kinase in synaptosomal cytosol. This kinase catalyzes the phosphorylation of several endogenous neuronal proteins. The protein kinase and major substrates bind to CaM-Sepharose in a calcium-dependent manner, and can be eluted by addition of EGTA to the eluent. Affinity chromatography using CaM-Sepharose results in a 20-fold purification of the enzyme. However, additional procedures result in a loss of enzyme activity. Therefore, we studied the stability of this protein kinase under various conditions. It appears that there are two different mechanisms involved in loss of enzyme activity. One is calcium-dependent and results in loss of total enzyme, the other is calcium-independent and results in loss of calmodulin sensitivity.

575

Objectives: Calcium ion, through the activation of a specific calcium-dependent protein kinase, has been found to regulate the phosphorylation of specific proteins found in preparations of synaptic plasma membranes, synaptic vesicle membranes, cerebral cortical slices, synaptosomes and synaptosomal lysates. In some cases it has been shown that this calcium-dependent phosphorylation requires the heat-stable calcium binding protein, calmodulin. Since the calmodulin-regulated protein phosphorylation of synaptic vesicle proteins has been linked to the release of neurotransmitter from isolated vesicles, the protein phosphorylation system may play an important role in neuronal function.

In order to fully characterize this Ca^{2+}, CaM-dependent kinase we are attempting to purify the enzyme and its substrates. This enzyme appears to be highly unstable, thereby making purification impossible. We, therefore, have studied the stability characteristics of this protein kinase in an attempt to determine the conditions necessary for stabilization.

Methods: The net incorporation of phosphate into cytosolic proteins was determined using one of two different assays. For samples to be subjected to gel electrophoresis: cytosol was incubated at 30°C for 2 minutes in the presence of HEPES, pH 7.0, 50 mM; $MgCl_2$, 10 mM; γ^{32}P-ATP, 5 µM and in some cases $CaCl_2$, 50 µM; calmodulin, 1 µg. The reaction was terminated by solubilizing the cytosol proteins in 100 µl of a sample buffer containing 9% SDS, 0.03 M Tris HCl buffer, pH 8.0, 6% β-mercaptoethanol, 3 mM EDTA and 27% sucrose. For determination of kinase activity after column chromatography: aliquots of fractions were incubated under the same conditions as above. The reaction was terminated with 1 ml 20% cold TCA. The samples were put over millipore filters and washed twice with 10% TCA.

Proteins were resolved on SDS-polyacrylamide slab gels. The acrylamide concentration was 6% and 10% in the stacking and resolving gels, respectively. Both stacking and resolving gels contained 0.1% SDS. Following electrophoresis the gels were fixed and stained with 0.1% Coomassie blue R250 in 50% methanol, 10% acetic acid. Gels were then destained and dried under heat and vacuum. Phosphorylated proteins were located by autoradiography using Kodak RP x-ray film.

Calmodulin-Sepharose was prepared by standard methods of ligand binding to cyanogen bromide-activated Sepharose. Synaptosomal cytosol was put over a calmodulin-Sepharose column in a buffer containing HEPES, pH 7.0, 20 mM; β-mercaptoethanol, NaN_3, 1 mM and NaCl 300 mM. Proteins binding to calmodulin were eluted with the same buffer with 1 mM EGTA substituted for $CaCl_2$.

Major Findings: Storage of the lysate at 4°C resulted in decreasing amounts of basal and CaM-activated protein kinase activity bound to CaM-Sepharose. After 24 hours of storage the amount of enzyme activity retained by the gel was approximately 50% of that bound at the zero time point, and after 48 hours only negligible kinase activity was retained.

If the crude synaptosomal pellet was lysed into buffer containing 1 mM Ca^{2+}, only a trace amount of basal or CaM-activated protein kinase activity was retained by CaM-Sepharose.

The stability of the protein kinase which had been partially purified on CaM-Sepharose was also investigated. CaM-Sepharose eluates were stored in the presence or absence of Ca^{2+}. In the absence of Ca^{2+}, basal protein kinase activity remains constant for 48 hours after elution, whereas CaM-activation of the protein kinase

is reduced to approximately 50% of its initial activity after 24 hours and to 30% after 48 hours of storage. If the CaM-Sepharose eluates are immediately adjusted to 1mM Ca^{2+}, the partially purified protein kinase rapidly loses activity, much like the crude preparation lysed in the presence of Ca^{2+}. After only 24 hours of storage at 4°C the amount of CaM-activated protein kinase activity was reduced to less than 10% of the fresh preparations. The addition of CaM to the eluate fraction did not enhance the stability of the enzyme in either the presence or absence of Ca^{2+}. These results indicate that the partially purified protein kinase is also quite unstable and that the destabilization is enhanced by Ca^{2+}.

The CaM-Sepharose eluates of cytosol were subjected to SDS-polyacrylamide gel electrophoresis to determine if the molecular weights (MW) of any of the eluted proteins had changed during storage. Eluates contained two major proteins with MW's of 100,000 and 77,000 daltons. Storage at 4°C altered the protein staining pattern substantially. Concurrent with a decrease in binding of the 100,000 and 77,000 MW proteins, two additional protein bands of 46,000 and 36,000 MW appeared in the eluate fraction.

Significance to Biomedical Research and Institute Program: Specific phosphorylated proteins have been implicated as physiological effectors for the diverse actions of a variety of intracellular regulatory agents. The calcium-regulated phosphorylation system in neuronal cytosol may play an important role in synaptic function as indicated by the observation that soluble enzymes involved in transmitter synthesis are activated under phosphorylating conditions. The rapid loss of Ca^{2+}, CaM-dependent protein kinase activity and loss of sensitivity to CaM creates problems for further purification and characterization of the role of this enzyme in synaptic function.

Proposed Course of Project: Further studies will be done to determine the mechanism of loss of activation by calcium and CaM. These studies will include the role of proteases in the degradation of the protein kinase and other means to increase the stability of the enzyme. Once this has been accomplished, several methods will be used, in addition to CaM-Sepharose chromatography, in order to purify this Ca^{2+}, CaM-dependent protein kinase e.g. DEAE, ATP-Sepharose and molecular sieve chromatography and nondenaturing gel electrophoresis. Substrates will be chromatographed and identified by two-dimensional gel electrophoresis. The characteristics of this kinase as well as the kinase-substrate interactions will then be investigated in an attempt to determine the physiological significance of these phosphoproteins.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03511-03 HE

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

The Role of the Brain Serotonergic Neuronal System in Blood Pressure Regulation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Donald M. Kuhn	Staff Fellow	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharm.	HE NHLBI
	William A. Wolf	Guest Worker	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.7	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Microinjection of serotonin directly into the nucleus tractus solitarius produced a dose dependent increase in arterial blood pressure of anesthetized rats. The serotonin antagonists BOL and metergoline significantly attenuated the serotonin pressor effect and the serotonin uptake inhibitor fluoxetine significantly enhanced the magnitude of the pressor response. Electrical stimulation of the dorsal raphe nucleus or microinjections of serotonin into the preoptic region of the hypothalamus produced a transient rise in arterial blood pressure of both spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) controls. These effects can be attenuated by metergoline. Injections of 5-hydroxytryptophan (5HTP), the immediate precursor of serotonin in brain, lowers blood pressure in the SHR but it appears that this effect is not related to the conversion of 5HTP to serotonin in brain.

574

PHS-6040
(Rev. 2-81)

Objectives: The purpose of the present experiment was to investigate the role of the neurotransmitter serotonin in modulating arterial blood pressure by microinjecting the amine into brain sites known to exert powerful influence over blood pressure (hypothalamus, nucleus tractus solitarius) or by electrically stimulating the dorsal raphe nucleus in normotensive and genetically hypertensive rats. Furthermore, the serotonin precursor 5-hydroxytryptophan was injected systemically into rats to study the effects of increases in brain serotonin on blood pressure by a less "invasive" method.

Methods: Male Sprague-Dawley rats, spontaneously hypertensive, and Wistar-Kyoto rats were used. Rats were anesthetized with chloropent and one femoral artery and one femoral vein were cannulated. The subjects were then mounted in a Kopf stereotaxic instrument for stimulation. Twisted bipolar electrodes (0.25 mm diameter) were aimed at the dorsal raphe and electrical current was delivered via a WPI dual channel pulse stimulator using stimulus isolation units to ensure delivery of a constant current stimulus. The following parameters were varied at the indicated values: frequency (1, 10, 25, 50 Hz), current (100-500 uA), pulse duration (0.1-1.0 msec). Alternating biphasic squarewave pulses were delivered in trains lasting for 5 sec. Blood pressure was measured with a Statham P23ID transducer and recorded on a Sanborn Polygraph. After stimulation experiments brains were fixed in formaldehyde and electrode placement was verified histologically. Microinjections into brain structures were delivered under stereotaxic control. Injections were delivered in a volume of 0.5 ul over a period of 10-30 secs. 5-hydroxytryptophan was injected intraperitoneally in a volume of 1 ml/kg.

Major Findings: Direct microinjections of serotonin (2, 7.5, 15, 30 nmol) into the NTS of anesthetized normotensive rats produced rapid and short-lived (< 15 min) increases in blood pressure ranging from 4-20 mm Hg. Heart rate did not change systematically after serotonin injections. Pretreatment of animals with the specific serotonin uptake inhibitor fluoxetine significantly increased the magnitude of the serotonin pressor effect but did not prolong it. The serotonin receptor antagonists BOL and metergoline were effective in blocking the serotonin pressor effect.

Electrical stimulation of the dorsal raphe nucleus in SHRs also produced profound effects on blood pressure. For example, increases as large as 45 and 65 mm Hg in WKY and SHR respectively, were observed after stimulation at 500 uA. Blood pressure increased rapidly with the onset of stimulation and fell rapidly upon termination of the stimulus train. At the highest current intensities (400 and 500 uA), SHRs responded to electrical stimulation with significantly greater increases in blood pressure much like that observed in normotensive rats. The pressor response to serotonin in both SHR and WKY could be blocked by the serotonin antagonist metergoline.

Injections of 5-HTP produced a small decrease in blood pressure. When 5-HTP treatment is combined with fluoxetine the hypotensive effects of 5HTP are accentuated.

Significance to Biomedical Research and Institute Programs: These results extend previous studies (Kuhn et al., 1980, Hypertension 2:243-255) which established that the brain serotonergic neuronal system constitutes an important central pressor network. These data also integrate well with a growing body of evidence which has recognized the potential importance of brain serotonin in the regulatin of the cardiovasuclar system and in the expression of certain types of hypertension.

These experiments continue to provide an impetus for the investigation into the pharmacologic and brain physiologic regulation of blood pressure. Furthermore, while serotonin precursors often produce effects on blood pressure which are opposite to those of serotonin, their usefulness as antihypertensive agents should be further investigated.

Proposed Course of Project: The following experiments will be undertaken to delineate the anatomical and neurochemical determinants of hypertension within the serotonergic neuronal system. Some of these experiments are in progress:

1. Discrete chemical lesions of the brain serotonin sytem will be induced with the neurotoxin 5, 7-DHT and the development of renal hypertension (DOC-salt) will be monitored.

2. The affects of increases in central nervous system serotonin on circulating renin activity will be determined.

3. The "functional" neuroanatomy of the serotonin pressor effects will be investigated by electrically stimulatng the dorsal raphe nucleus while ^{3}H-2-deoxyglucose is infused.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 03513-03 HE

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Sodium-sensitive cocaine binding to rat striatal membranes (Revised title)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I. :	Linda Kennedy	Guest Worker	HE	NHLBI
Other:	Ingeborg Hanbauer	Pharmacologist	HE	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.6	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project has emphasized the characterization of the cocaine binding site in rat striatal membranes. Binding is rapid, saturable, reversible and dependent on tissue concentration. In striatal membranes, sodium induces a two-fold increase in the maximal number of binding sites without altering their affinity for cocaine. This stimulatory effect is specific to sodium, concentration-dependent, and occurs in membranes prepared from striatum but not from other brain regions. Sodium-dependent binding is eliminated following 6-hydroxydopamine-induced lesions of the striatum, suggesting that the binding site is localized presynaptically on dopaminergic nerve terminals. This finding in conjunction with experiments determining the ability of various drugs to compete for the cocaine binding site, suggests that the sodium-sensitive binding site is related to dopamine uptake sites in striatum. However, the sodium sensitivity does not appear to be related to the presence of an endogenous inhibitor of cocaine binding, since this factor is present not only in striatum, but also in brain regions not demonstrating sodium-stimulated binding.

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Objectives

Recent work in this laboratory has demonstrated that the administration of cocaine to rats produced a sensitization of striatal dopamine receptors (Memo et al., Neuropharmacology 20:1145, 1981). It was therefore of interest to determine the mechanism by which cocaine produced its effect on the dopamine receptor. A novel approach to this problem was suggested by a report of saturable cocaine binding to membranes prepared from mouse brain (Reith et al., Life Sci. 27:1055, 1980). We therefore began a characterization of cocaine binding to rat striatal membranes.

Methods

^{3}H-cocaine binding was measured by incubating crude membrane preparations in the presence of ^{3}H-cocaine and other agents for 20 minutes at room temperature. The reaction was terminated by rapid filtration of the sample over Whatman GF/B filters. Filters were washed 3 times with buffer, dried, and counted to determine bound radioactivity.

The endogenous inhibitor of cocaine binding was prepared by homogenizing tissue in 1% NaCl, centrifuging, and heating the resulting supernatant at 100°C for 15 minutes. After re-centrifugation, the supernatant fraction served as the source of the endogenous inhibitor.

Major Findings

This work has demonstrated that cocaine binds to membranes prepared from rat striatum, cerebellum, hippocampus, olfactory tubercle, hypothalamus, and frontal cortex. Binding is rapid, reversible, saturable and dependent on tissue concentration. Incubation of striatal membranes with 50 mM NaCl increases the maximal number of binding sites approximately 2 fold without altering the affinity of the binding site for cocaine. This stimulatory effect is specific to sodium, since 50 mM NaF and Na acetate also enhance binding, whereas equimolar concentrations of several salts which do not contain sodium are inhibitory. Furthermore, the stimulatory effect of NaCl is concentration-dependent, and occurs in membranes prepared from striatum but not from other brain regions.

The sodium-sensitive binding site appears to be localized presynaptically on dopaminergic terminals, since 6-hydroxydopamine lesions, which destroy these terminals, markedly attenuate cocaine binding in the presence of NaCl. In this study, the amount of binding occurring in the presence of NaCl is strongly and positively correlated to striatal dopamine concentration. In contrast, striatal kainic acid lesions, which destroy postsynaptic cell bodies, do not alter binding of cocaine. These data suggest that in the presence of sodium, cocaine binds to dopamine uptake sites. This suggestion is further supported by the sodium dependency of catecholamine uptake and by data demonstrating that agents which inhibit dopamine uptake are potent inhibitors of cocaine binding, whereas norepinephrine and serotonin uptake inhibitors and other agents are less effective.

A second facet of these studies has focused on the characterization of an endogenous inhibitor of cocaine binding initially described by Reith et al. (Neurochem. Res. 5: 129, 1980). Incubation of rat brain membranes with heat-treated extracts of striatum results in a concentration-dependent inhibition of cocaine binding. Extracts prepared from brain regions not demonstrating sodium-stimulated binding also inhibit cocaine binding to striatal membranes. These data show that in striatal membranes, cocaine binding is sensitive to both

sodium and to an endogenous inhibition. However, these phenomena can be dissociated, since the inhibition is present even in tissues that do not demonstrate sodium-stimulated binding.

Proposed course of project

Plans for future research include the following: 1) Rats will be treated with cocaine both acutely and chronically, and the maximal number of binding sites and affinity of cocaine binding measured. This experiment will provide evidence concerning the in vivo regulation of the cocaine binding site. 2) The relationship between cocaine binding and a physiological process known to be affected by cocaine, dopamine uptake into striatal synaptosomes, will be investigated. The effect of the endogenous inhibitor on dopamine uptake in the presence and absence of cocaine will also be studied. 3) A series of experiments will attempt the isolation and biochemical characterization of the inhibitor of cocaine binding. Kainic acid and 6-hydroxydopamine will be used to determine the cellular localization of the inhibitor. Finally, the inhibitor will be quantified in naive animals and in animals chronically treated with cocaine to determine whether levels of the inhibitor can be regulated in vivo.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03514-03 HE

PERIOD COVERED

October 1, 1981 to September 31, 1982

TITLE OF PROJECT (80 characters or less)

Heat Stable Cytosolic Factors Affecting Synaptic Membrane Phosphorylation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Judith Juskevich	Staff Fellow	HE NHLBI
Other:	Donald Kuhn	Staff Fellow	HE NHLBI
	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A variety of effector-specific protein kinases have been identified in brain tissue and, most recently, a cyclic nucleotide-independent protein kinase has been the subject of much interest. This particular protein kinase is stimulated by phospholipids in the presence of calcium. This phospholipid-sensitive kinase can apparently be distinguished from a calmodulin (CaM)-activated protein kinase on the basis of substrate specificity. However, it has not been demonstrated with certainty that the phospholipid- and CaM-activated protein kinase are distinct enzymes, nor has a phospholipid-specific protein kinase been demonstrated. We have demonstrated that the activation of protein kinase by phospholipids can be mimicked by sodium dodecylsulfate (SDS), as well as the hydrophobic probe 8-anilino-1-napthalene-sulfonate (ANS), indicating that the phospholipid effect on protein kinase represents a more general stimulation of enzyme activity than previously indicated.

584

Objectives: Several different protein kinases have been identified in neuronal tissue. We are studying various factors that activate both membrane-bound and soluble protein kinases in an attempt to determine their involvement in regulation of protein phosphorylation.

Methods: Sprague-Dawley rats were decapitated and the cortex removed and homogenized. The homogenate was centrifuged at 100,000 x g for 60 minutes and the resulting supernatant used as the source of protein kinase.

CaM-deficient cytosol was prepared by two methods: 1) cytosol was loaded onto a DEAE column and the protein kinase was eluted along with substrate proteins; 2) an aliquot of cytosol was loaded onto a fluphenazine-Sepharose column and the effluent from the column was used as the source of protein kinase and substrates.

Phosphorylation of cytosolic proteins was assayed at 30°C in a mixture containing 50 mM HEPES, pH 7.0, 10 mM or 1 mM $MgCl_2$, 1 mM DTT, 5 μM γ ^{32}P-ATP, in the absence or presence of 50 μM $CaCl_2$ and 1 μg calmodulin. After 1 minute the reaction was terminated by the addition of 100 μl of an electrophoresis sample buffer.

Proteins were than resolved on SDS-polyacrylamide slab gels. The phosphorylation of specific synaptic proteins was determined by autoradiography.

Major Findings: As previously reported, CaM stimulates the phosphorylation of various endogenous proteins with the major effect on proteins with molecular weights (MW) of 50,000 (50K), 55,000 (55K), and 60,000 (60K) daltons. The effects of phosphatidylserine (PS) and phosphatidylinositol (PI) primarily stimulated the phosphorylation of a protein with a MW of 47,000 (47K) daltons, while somewhat smaller effects were observed on two proteins with higher MW (75K and 77K daltons). SDS had an effect similar to the phospholipids in that it predominately stimulates phosphorylation of proteins with MW of 47K and 77K. These effects of CaM, phospholipids and SDS were Ca^{2+}-dependent.

Addition of the hydrophobic probe ANS blocked the stimulation of protein phosphorylation by CaM but not by PS or SDS. ANS itself caused a Ca^{2+}-dependent stimulation of phosphorylation. ANS enhanced the phosphorylation of protein bands with approximate MW's of 75K and 47K daltons, much like the effects produced by phospholipids and SDS.

SDS blocks the CaM-stimulated increase in phosphorylation, while addition of PS resulted in an additive effect, i.e., increased phosphorylation of proteins affected by CaM and phospholipids.

Significance to Biomedical Research and Institute Program: Phosphorylation of specific proteins found in neuronal membranes and neuronal cytosol may be involved in modulating synaptic function. Therefore, studying the regulation of protein phosphorylation may result in a greater understanding of the mechanisms involved in the regulation of synaptic function. In this study we have looked at two factors, CaM and phospholipids, that affect protein phosphorylation. The differences in their effects and possible mechanisms of action have been discussed.

Proposed Course of Project: The mechanism of activation of protein kinases by various factors is still unclear, as is the interaction of the different kinase

systems. In particular, it is uncertain whether the phospholipid-sensitive protein kinase is an enzyme distinct from the Ca^{2+}, CaM-dependent enzyme. Future work on this project will be concerned with identifying the mechanism of activation of phosphorylation by phospholipids. These studies will include determining the structure-activity relationship of various phospholipids in comparison to compounds that have similar detergent or ionic properties. The second aspect of the project will be concerned with determining whether the phospholipid-sensitive protein kinase is separate and distinct from the Ca^{2+}, CaM-dependent kinase. In order to do this it will be necessary to purify the Ca^{2+}, CaM-dependent kinase in a state where it still maintains CaM-sensitivity. The purification of the kinase will be attempted using a variety of chromatographic methods including affinity chromatography (CaM-Sepharose and ATP-Sepharose), molecular sieve and DEAE chromatography and non-denaturing polyacrylamide gel electrophoresis.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03516-03 HE

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

The Biosynthesis, Distribution and Biological Role of Substance P

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Mei-Lie Swenberg	Research Chemist	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharm.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0		1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The developmental patterns of Substance P neurons in the central nervous system of rats has been examined by determining the SP concentration in various brain regions of prenatal and neonatal animals.

The possible interaction of SP neuronal components with the endocrine system was investigated in pregnant, hypophysectomized and sphlanchnicectomized rats. Significant alteration in SP content was observed by neuroendocrine manipulation. In an effort to further probe the molecular mechanism of action for substance P an anti idiotypic antibody was raised against anti-Substance P immunoglobulin.

PHS-6040
(Rev. 2-81)

Objectives: The major, overall, objective of this project is to further understand the role of Substance P as a neurotransmitter and neuromodulator, as well as a possible hormonal substance. The specific objectives for this year are:

1) Conduct an ontogenic analysis of substance P in the central nervous system of the rat.

2) Prepare purified SP antibodies and attempt to raise anti-idiotypic antibodies against anti substance P.

3) Examine the effect of neuroendocrine manipulation on Substance P content in central and peripheral tissues.

4) Attempt to study the role of Substance P in cardiovascular regulation in normotensive and genetically hypertensive rats.

Methods: SP is measured by radioimmunoassay with SP labeled with Bolten and Hunter reagent [N-succinimidyl-3-(4 hydroxy, 5-[^{125}I] iodophenyl) propionate]. Antibodies have been generated in New Zealand white rabbits by microimunization with Bovine serum albumin conjugated SP (BSA-SP) (for anti SP) or purified anti SP immunoglobulin (for idiotypic antibody of anti SP).

Short term tissue cultures are carried out using brain cells from various brain regions, e.g. substantia nigra, caudate, hypothalamus, and cerebellum of mature rats and the mid brain and hypothalamus of embryonic rats at different stages of development. Affinity column, acrylamide gel electrophoretic, high pressure liquid chromatographic, NMR and other spectroscopic methods have been applied in purification, and identification of precursors, metabolites, antibodies and SP. The blood pressure of animals are measured by a tail cuff method.

Major Findings: Substance P can be detected in rat brain as early as the 11th day of gestaton. In most brain regions, SP continuously increased in amount until the day of birth. However, in cerebellum and forebrain the total amount of SP reached its highest level prior to birth, the concentration of SP (pg/mg protein) reached the maximum plateau on the 15th-16th day of gestation. Upon birth the total amount and concentration of SP decreased dramatically in all brain areas. In cerebellum and forbrain, the concentration remained at the low level for 1 week, but in the midbrain and hypothalamus, a continued decrease in concentration was observed, while the total SP (pg) increased in all areas, because of the rapid growth of the CNS. This observation suggests that SP might play a different role during the course of embryonic development or that a change in physiological function takes place upon birth.

Anti idiotypic antibody raised against anti substance P immunoglobulin binds to SP antibodies and displaces bound SP as does SP. It will be determined whether this antibody can activate the SP receptor.

Bilateral splanchnicectomy caused a lowering of the SP level in all brain regions studied, except in the adrenal gland, which showed a slight increase in SP. Hypophysectomy significantly elevated the SP level in the hypothalamus but lowered the level in the substantia nigra (SN). Three possible projections of SP neurons can be speculated from the summarized results: 1) from splanchnic nerve to CNS; 2) hypothalamus to hypophysis; and 3) hypophysis to substantia nigra.

Pregnancy causes higher SP levels in most of the brain regions studied, (hypophysis, hypothalamus and SN), but not in the caudate putamen. In pregnant rats (14-15 day gestation) SP was also detected in the kidney at a significantly higher level (1348 ± 300 pg/mg protein) than was found in the controls (1.3 ± 0.5 pg/mg protein).

Current results also suggest that the neuroendocrine system has an important impact on SP in both central and peripheral systems. It is possible that corticoids affect the synthesis of SP and SP may play an important role in pregnancy and embryonic development.

Significance to Biomedical Research and Institute Programs: The peptide Substance P appears to be an important neurotransmitter in the CNS. Previous studies in our laboratory have demonstrated the nature of the interaction of this neuronal system with the dopamine and serotonin neuronal systems. The current experiments were designed to further understand both the development of the SP system and its interaction with some of the endocrine system. The work is directed at understanding the role of SP in cardiovascular control and other physiologic systems.

Proposed Course of Project: The rather broad approach of the past year will give way to the investigation of some specific questons:

1. An attempt will be made to isolate and characterize the precursor and the enzymes responsible for the generation of SP.

2. The SP receptor will be studied in pregnant and prenatal animals.

3. The anti idiotypic antibody will be characterized from both a molecular and receptor active approach.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03518-02 HE

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Serotonin Receptors and Cardiac Ventricular Sarcolemma

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Elliott Kulakowski	Staff Fellow	HE NHLBI
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COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A high affinity serotonin receptor has been identified on the cardiac ventricular sarcolemma. Serotonin analogues compete for this binding site but classical serotonin antagonists are weak inhibitors. At high concentrations, catecholamines are able to displace serotonin binding but this inhibition does not appear to be related to serotonin binding to adrenergic receptors.

590

PHS-6040
(Rev. 2-81)

Objectives: Serotonin (5-HT) exerts a direct, positive inotropic effect on the rat heart. In order to determine the mechanism by which serotonin brings about this response, we investigated the interaction of serotonin with the cardiac sarcolemma. The studies consisted of: 1) examining for the presence of a discrete serotonin receptor, 2) studying the effects of serotonin on sarcolemmal enzymes, and 3) determining if serotonin plays a role in ion movement across the sarcolemma.

Methods: Sarcolemma Preparations. Male Sprague Dawley rats (200-250 gms) were used in all experiments. They were housed at the N.I.H. animal facilities and given food and water ad libitum. The hearts were removed from the rats and perfused retrogradely, within 60 seconds, with 0.25 M ice cold sucrose to remove blood from the ventricles and coronary vessels. The ventricular tissue was isolated, weighed and minced in 5 volumes (w/v) 0.25 M ice cold sucrose. The tissue was homogenized at 4°C in a Waring blender and centrifuged at 600 x g for 10 min. The pellet was washed three times by suspension in 10 mM Tris-HCl, pH 8.0 and centrifuged as described above. The final pellet was suspended in buffer and 4.0M LiBr was added slowly, with stirring, to yield a final concentration of 0.4M LiBr. The suspension was stirred for 1 hr at 4°C, diluted four-fold with buffer and passed through eight layers of cheese cloth. The filtrate was centrifuged at 2000 x g for 10 minutes at 4°C and the pellet washed with 10 mM Tris-HCl, pH 7.5 containing 25% KBr and centrifuged for 30 minutes at 7000 x g. The pellet was washed three times with 10 mM Tris HCl, pH 8.0 and the final pellet was suspended in 10 mM Tris HCl, pH 7.4 containing 120 mM NaCl, 1.25 mM $CaCl_2$, 4.8 mM KCl, 1.2 mM $MgSO_4$ and 1.2 mM KH_2PO_4.

Binding Assay. The binding of 5-hydroxytryptamine to the isolated cardiac ventricular sarcolemma was determined using freshly prepared membranes. Membrane (80-100 ug sarcolemma protein) was preincubated at 24°C for 15 minutes in the ion-containing buffer prior to the addition of [^{3}H]-serotonin (32.5 Ci/nmol). The reaction was terminated after one-hour incubation by the addition of 3 ml ice cold buffer. The membranes were isolated by filtration on Whatman GF/B glass fiber filters and washed three times with 3 ml volumes of ice cold buffer. The filters were placed in scintillation fluid and counted in a Beckman LS-8100 liquid scintillation counter. Specific binding was determined as the difference between total binding and non-specific binding, which was determined in the presence of at least 1000 fold excess of serotonin.

Major Findings: A discrete serotonin receptor is present on the rat cardiac ventricular sarcolemma. Binding of serotonin over the concentration range 1-150 nM was non-cooperative as determined from the Hill coefficient of 0.93. Scatchard analysis of the binding data reveals a dissociation constant (K_D)=48.8 nM and a Bmax=12.2 p moles/mg sarcolemma protein. This data compares favorably with a K_D=45.6 nM determined the rate constants for association and dissociation.

Serotonin binding to cardiac ventricular sarcolemma is inhibited by serotonin analogues but not by the classical anti-serotonin agents. The order of potency for the serotonin analogues is 5-hydroxyindoleacetic acid > 7-dihydroxytryptamine > 5-hydroxytryptamine > 5-methoxytryptamine > tryptophan > tryptamine. Based on this order of potency the 5-hydroxy moiety appears to be necessary for binding. In addition to LSD and its analogues, cyproheptadine, methiothepin and cinanserin fail to appreciably alter serotonin binding while mianserin only inhibits binding

at 40% at 2000 fold excess.

Catecholamine, at a concentration greater than 10^{-7}M, exhibits cross reactivity for the cardiac 5-HT binding site. The adrenergic agents isoproterenol, epinephrine, nonepinephrine and phenylephrine have IC_{50} values of 7, 40, 60 and 100 uM respectively when compared to 48 mM serotonin. However, this inhibition by catecholamines appears to be unrelated to either α- or β-adrenergic receptors since neither propranolol nor phentolamine inhibit serotonin binding. Furthermore, if serotonin acted through the β-adrenergic receptor, then 5-HT should stimulate adenylate cyclase. However, no activation of adenylate cyclase was observed.

The serotonin receptors on rat heart sarcolemma are observed over the same concentration range that is necessary to elicit a positive inotropic effect. The action of 5-HT is not mediated through α- or β-adrenergic receptors and is not related to cAMP production. However, serotonin may function through activation of the sarcolemma ATPases or alter calcium binding or flux in the heart. These parameters are currently being investigated.

Significance to Biomedical Research and Institute Programs: Serotonin is synthesized and stored in high concentrations in the heart similar to the catecholamines. Thus, since both serotonin and catecholamines produce a positive inotropic response they may both play an important role in the stressed myocardium. Our results indicated that: 1) serotonin binds to discrete serotonin receptors, 2) these binding sites are not related to adrenergic receptors, 3) since it does not activate adenylate cyclase its action may be on other membrane proteins or related to ion movements.

Little is known about the interaction of 5-HT with the sarcolemma. Even less is known about the mechanism by which serotonin induces a positive inotropic response. A better understanding of the mode of action of 5-HT and perhaps suggest new treatments for the stressed myocardium.

Proposed Course of Project: An examination of the effects of serotonin on sarcolemmal proteins and calcium movements will be investigated. Various concentrations of serotonin will be added to membrane preparations to see if there is a change in $Na^{+}K^{+}$-ATPase, Mg^{+2}-ATPase, or Ca^{+2}-ATPase. In addition, calcium binding and uptake experiments are proposed to determine if serotonin has any effect on calcium movements across the sarcolemma. Any stimulatory effect of calcium will be characterized in the presence and absence of calcium antagonists such as verapamil and lanthanum chloride.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03519-02 HE

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Analysis of 6-Phenylpterin as a Cofactor for Various Hydroxylases from Mammalian Tissue

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Leonard Miller	Guest Worker	HE NHLBI
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	Donald Kuhn	Senior Staff Fellow	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.25	0.25	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Tetrahydropterin forms the basic structure of a series of analogues which serve effectively as cofactors for hydroxylase enzymes. Most analogues are formed by placement of various side chains on the 6-carbon. We have recently acquired a compound which has a phenyl group attached at this 6-position. The present investigation compared this compound with tetrahydrobiopterin for effective hydroxylation of phenylanine, tyrosine and tryptophan. The enzymes used were: phenylalanine hydroxylase purified from rat liver and tyrosine and tryptophan hydroxylase from rat brain. In addition we examined the ability of the reductase system to continually regenerate reduced 6-methylpterin. Our kinetic analysis showed that 6-phenylpterin is as effective as the natural cofactor (tetrahydrobiopterin) for the various hydroxylases. Also, 6-phenylpterin is incorporated effectively into the endogenous reductase system.

593

Objectives: As the rate-limiting enzyme in catecholamine synthesis in the central nervous system, tyrosine hydroxylase is intimately involved in the regulation of neurons utilizing catecholamine neurotransmitters. Over the past few years numerous investigations have focused on the regulatory mechanisms of this enzyme to determine if the synthesis of catecholamines could be manipulated in vivo. Because it has been speculated that the enzyme has limiting amounts of its cofactor we attempted to influence synthesis by administering cofactor analogues which had a high probability of crossing the blood-brain barrier and hopefully accumulating in the synaptoplasm. A compound which was brought to our attention and which had a high probability of accomplishing these goals was 6 phenyltetrahydropterin ($6PPH_4$). Our initial experiments concentrated on the effectiveness of 6 PPH_4 on the hydroxylase process in vitro. 6-Methyltetrahydropterin and tetrahydropterin were used. The kinetic constants K_m and V_{max} were obtained from the x and y intercepts of double-reciprocal plots.

Methods: $6PPH_4$ was obtained from Dr. Carl Storm at Howard University. It was stored dessicated and when used dissolved in 0.1 N HCL with determination of its concentration by measuring its O.D. at 264 nmeters with an E = 16,000. We subsequently determined the activity of the three hydroxylase enzymes: tyrosine (TH) phenylalanine (PH) and tryptophan hydroxylase (Try H). TH was obtained from rat striatum. Briefly, striata were removed from the rats, homogenized in 0.5 M KP, buffer pH 6.0 (containing 0.2% TY-100) and centrifuged at 18,000 rpm for 30 min. The supernatant was decanted and immediately passed over a Sephadex G-25 column for removal of endogenous catecholamines. Enzyme activity was determined using 3,5 H^3 tyrosine and measuring the amount of labeled water formed. PH hydroxylase was purified from rat adrenal tissue by our standard laboratory method. Enzyme activity was determined using labeled phenylalanine and measuring the amount of labeled water formed. Try H was determined using supernatants from homogenized rat midbrain tissue. Activity was determined at pH 7.2 using 50 mM Tris-HCl and incubating for 15 min. The amount of product formed during the enzymatic reaction was determined by fluoresence using excitation-emission wavelengths of 295-540 nm and calculated from a standard curve of 5-HTP run in parallel with each assay. All enzyme activities were determined using various concentrations of 6 PPH_4 for comparison.

Major Findings: Our analyses of the different hydroxylases show that 6 PPH_4 is as efficient a cofactor as the natural form, BH_4, while the pterin analogue, $6MPH_4$ shows the highest K_m value. (See table) Also, the PH assay procedure uses the reductase system for constant regeneration of reduced cofactor. $6PPH_4$ can be effectively reduced by this enzyme in addition to serving as a cofactor for the hydroxylase. Determination of V_{max} values also confirms that 6 PPH_4 is almost as effective as the natural cofactor.

Significance to Biomedical Research and Institute Programs: The present results form the first step in confirming the potential for the use of $6PPH_4$ in future studies. This pterin analogue serves effectively as a cofactor. The next area of investigation to focus on is the administration and penetration of this compound across the blood-brain barrier.

Proposed Course of Project: We plan to obtain additional lipophilic analogues that potentially cross the blood-brain barrier and to establish their kinetic characteristics in the various hydroxylase systems.

Analysis of Pterin Analogues on Kinetic Parameters of Tyrosine, Tryptophan and Phenylalanine Hydroxylases

	K_m (αM)		
Enzyme	Biopterin-H_4	6mPH_4	6-ϕ Pterin-H_4
Tyrosine Hydroxylase	200	144	25
Tryptophan Hydroxylase	14	108	4
Phenylalanine Hydroxylase	0.40	31	0.8

	V_{max}		
	Biopterin-H_4	6mPH_4	6-ϕ Pterin-H_4
Tyrosine Hydroxylase (pmolesμl5 min)	15	388	93
Tryptophan Hydroxylase (nmolesμmin)	0.15	0.58	0.34
Phenylalanine Hydroxylase (nmolesμ45 min)	0.38	3.88	0.78

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03520-02 HE

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Dopamine Receptor Regulation in Schizophrenic Illness

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Maurizio Memo	Guest Scientist	HE NHLBI
OTHER:	Ingeborg Hanbauer	Pharmacologist	HE NHLBI
	Walter Lovenberg	Chief, Sec. Biochem. Pharm.	HE NHLBI
	Joel Kleinman	Psychiatrist	NIMH

COOPERATING UNITS (if any)

Adult Psychiatry Branch, NIMH, St. Elizabeths Hospital, Washington, D.C.

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies on the regulation of adenylate cyclase in nucleus caudatus, nucleus accumbens, hippocampus and cerebellar cortex of control and schizophrenic brains were carried out. The adenylate cyclase activity in the dopamine-rich brain areas of schizophrenics was more enhanced by NaF, GTP or GppNHp than in controls. In contrast, these agents stimulated adenylate cyclase in cerebellar cortex and hippocampus of controls and schizophrenics to the same extent. The present results suggest that the regulatory subunit of G/F protein that facilitates adenylate cyclase activation is increased in dopamine-rich brain areas of schizophrenic subjects.

596

PHS-6040
(Rev. 2-81)

Objectives: Studies on the properties of adenylate cyclase-linked dopamine (DA) receptors were carried out in brain from schizophrenic subjects. In particular, the role of different regulatory components of adenylate cyclase, namely G/F protein and calmodulin (CaM), was studied in different DA-rich and DA-deficient brain regions from 12 non-schizophrenic (normal) and 10 schizophrenic subjects. Several reports on catecholamine receptors indicate that the regulatory component of G/F protein links the recognition site for neurotransmitter with adenylate cyclase. The event following the binding of the neurotransmitter and its recognition site is the release of GDP from G/F protein with subsequent binding of GTP. GTP bound to G/F protein activates adenylate cyclase. The rate-limiting step in the activation of adenylate cyclase seems to be the hydrolysis of GTP which is regulated by GTPase. G/F protein also has binding sites for fluoride ions. Fluoride bound to G/F protein is also able to stimulate adenylate cyclase.

The regulatory activity of calmodulin (CaM) has been suggested by data showing that 1) in the presence of Ca^{++}, CaM activates adenylate cyclase; 2) supersensitivity of DA-stimulated adenylate cyclase is associated with increased content of membrane-bound CaM, 3) subsensitivity of DA-stimulated adenylate cyclase is associated with a decreased content of membrane-bound CaM.

Methods: Adenylate cyclase activity was measured as previously described (Clement-Cormier et al., Proc. Nat. Acad. Sci., USA, 71 1113-1117, 1974). Incubation was carried out in presence or in absence of 4 different concentrations of NaF (1 mM, 2.5 mM, 5 mM, 10 mM) or GMP(NH)PP (5 uM, 10 uM, 50 uM, 100 uM). The amount of cyclic AMP formed was measured by radioimmunoassay.

CaM content was measured by enzyme-linked immunosorbent assay as previously described (Hanbauer et al.,Ann. N.Y. Acad.Sci. 356: 292-303, 1980).

Results: NaF- and Gpp(NH)p-stimulated adenylate cyclase activity in nucleus caudatus and nucleus accumbens was higher in schizophrenic than in normal subjects. In contrast, stimulation of adenylate cyclase by NaF in cerebellar cortex or hippocampus was similar in schizophrenic and normal subjects. Membrane-bound and cytosolic CaM content in nucleus caudatus and nucleus accumbens were similar in schizophrenic and normals. Our results strongly indicate a specific alteration in G/F protein regulation of adenylate cyclase in DA-rich brain areas of schizophrenic subjects. The enhanced response of adenylate cyclase to NaF or Gpp(NH)p may be interpreted as 1) increased affinity of the recognition sites for these agents; 2) increased number of the binding sites; 3) increased amount of G/F protein.

Proposed Course of Action: Studies are in progress to quantitate the amount of G/F protein in different brain regions from normal and schizophrenic subjects. Measurement of GTPase activity is planned to better characterize this alteration.

In view of the therapeutic action of some enkephalin-like peptides in schizophrenia, it would be of interest to see if these compounds interact with the coupling system of DA receptor.

Significance of Biomedical Research: This research project is designed to obtain a better understanding of biochemical defects that may exist in schizophrenic illness. Better insight on the abnormalities of dopaminergic transmission will allow more rational, specific and effective therapy.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03521-02 HE

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Regulation and Turnover of Tetrahydrobiopterin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Anne Culvenor	Guest Worker Fogarty International Center	HE NHLBI
OTHER:	Leonard Miller	Guest Worker	HE NHLBI
	Walter Lovenberg	Chief, Sec. Biochem. Pharm.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The rat cultured pineal gland was used to study tetrahydrobiopterin (BH4) regulation. Addition of the BH4 precursor, guanosine to the culture medium had no effect on levels of pineal [BH4 + quinonoid-dihydrobiopterin (qBH2)], [BH2 + biopterin (B)] or medium B measured 24 or 48 hours later. Addition of the putative precursor, sepiapterin increased pineal [BH2 + B] levels and decreased pineal [BH4 + qBH2] levels up to 2-fold and also markedly increased medium B (up to 7-fold) after 48 hours in a concentration-dependent manner. Medium B reflected either BH2 and/or B released from pineals, but did not reflect release of BH4, which was rapidly degraded in medium to pterin and non-fluorescent compounds. Addition of the dihydrofolate reductase (DHFR) inhibitor, methotrexate (MTX) to the medium in concentrations which completely inhibited pineal DHFR had little or no effect on pineal [BH4 + qBH2] whereas pineal [BH2 + B] and medium B increased up to 2-fold, suggesting that DHFR may be involved in the biosynthesis of BH4. A biopterin-bovine serum albumin conjugate is being used as an immunogen in rabbits to develop antibodies to biopterin, ultimately required for development of an immunoassay for biopterin.

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Objectives: The main aim of these experiments was to study the regulation of BH_4 in relation to monoamine synthesis. A minor aim was to develop an immunoassay for biopterin.

Methods: Pineals from male Sprague-Dawley rats were cultured for up to 48 hours. Oxidized and reduced B were assayed by differential iodine oxidaton and high performance liquid chromatography (HPLC) (Fukushima and Nixon, Anal. Biochem. 102, 176, 1980). DHFR activity in pineal homogenates was assayed by measuring the formation of [^{3}H]-tetrahydrofolate from [^{3}H]-dihydrofolate (Hayman et al. Anal. Biochem. 87, 460, 1978).

Major Findings: The effect of BH_4 precursors on pineal levels of reduced and oxidized B was studied to see if pineal BH_4 could be increased by this method. Since the known BH_4 precursor, GTP, does not cross cell membranes easily, intracellular levels of GTP were increased by culturing glands in the presence of 300 uM guanosine, a GTP precursor. Addition of guanosine to the culture medium had no effect on pineal levels of [BH_4 + qBH_2] or [BH_2 + B] after either 24 or 48 hours culture. Medium B was also unchanged at both times. These findings may reflect existing saturation of the rate-limiting enzyme in BH_4 biosynthesis, GTP cyclohydrolase, with substrate (GTP) or conditions of incubation did not result in increased levels of endogenous GTP.

Addition of the putative BH_4 precursor sepiapterin (1-34 uM) to the culture medium decreased pineal (BH_4 + qBH_2) and correspondingly increased pineal (BH_2 + B) up to 2-fold after 48 hours. There was also a marked increase (up to 7 times control) in medium B following culture of glands in sepiapterin. B in the medium was shown in separate experiments to reflect release of either BH_2 and/or B, but not of BH_4, which rapidly breaks down in the medium to pterin and non-fluorescent compounds. Therefore addition of sepiapterin to pineal cultures results in a large accumulation of BH_2, suggesting that pineal glands contain the enzyme sepiapterin reductase. The significance of the decreased pineal BH_4 in the presence of sepiapterin is unknown.

The effect of the potent DHFR inhibitor, MTX on reduced and oxidized B in pineals was investigated, since it has been proposed that DHFR may catalyze the formation of BH_4 from BH_2. Culture of pineals in MTX (0.5-10 uM; 6 and 24 hours) resulted in complete inhibition of DHFR activity measured in pineal homogenates. Addition of 0.5 or 5 uM MTX to the culture medium did not alter pineal levels of [BH_4 + qBH_2] after 6 or 24 hours, whereas 24 hour culture of glands in 10 uM MTX did result in a slight but significant drop of 30%. In contrast, pineal [BH_2 + B] increased to 150% of control after 6 hours culture in 0.5 or 5 uM MTX, and 150-250% of control after 24 hours culture in 10 uM MTX. Total B in medium increased to 150-200% of control following incubation of glands in MTX. The increase of pineal BH_2 in the presence of MTX, also mirrored by increased medium B, may reflect a build-up due to inhibition of DHFR. Although pineal BH_4 changed very little in the presence of MTX, this may reflect the very slow rate of BH_4 biosynthesis relative to its regeneration from qBH_2, catalyzed by the enzyme quinonoid dihydropteridine reductase (QDPR). Experiments in progress measuring the incorporation of [^{14}C]-guanosine into pineal BH_4 and BH_2 in the presence and absence of MTX should more clearly indicate whether DHFR is involved in BH_4 biosysthesis in the pineal.

Immunization of rabbits with the biopterin-bovine serum albumin conjugate of low epitope density has not so far yielded biopterin antibodies. A further series

of immunizations is being attempted using a different schedule of injections.

Significance to Biomedical Research and Institute Programs: The availability of model systems such as the rat cultured pineal gland for study of BH_4 regulation is very useful in gaining a further understanding of the mechanisms regulating BH_4 levels in monoaminergic neurons. These studies should clarify the important role of BH_4 in the regulation of monoamine systhesis.

Proposed Course of Project: The principal investigater terminates her fellowship at the end of August, 1982. Dr. Miller also terminates his stay as a Guest Worker. The experiments indicated above will be carried out, but this project will then terminate.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03522-02 HE

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Penetration of Biopterin and Other Analogues into Rat Brain Tissue and Their Effect on Endogenous Dopamine Synthesis Rates (Revised Title)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Leonard Miller	Guest Worker	HE NHLBI
OTHER:	Glenn Robinson	Summer Student	HE NHLBI
	Elliott Kulakowski	Staff Fellow	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	0.75	0.25

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The present investigation analyzed the ability of tetrahydrobiopterin (BH4) and 6-methyltetrahydropterin (6 mPH4) to penetrate into rat brain tissue following peripheral administration. The drugs were given i.p. at a dose of 100 mg/kg. Following injection of BH4 or 6 mPH4, 5 different areas of rat brain were removed at 5 different time periods to be analyzed for levels of BH4 and 6 mPH4: striatum, hippocampus, hypothalamus, cortex and cerebellum: time points - 0, 30, 60, 90, 120 and 150 min. post injection. At present we see no change in the endogenous levels of striatal BH4 following i.p. BH4. However, there is a significant increase in 6 mPH4 following its i.p. injection. We subsequently examined the effect of 6 mPH4 injection on endogenous dopamine synthesis by monitoring levels of the metabolites DOPAC and dopamine. There was only a small apparent effect of 6 mPH4 injection on DA and DOPAC.

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Objectives: As the rate-limiting enzyme in catecholamine synthesis in the central nervous system, tyrosine hydroxylase is intimately involved in the regulation of neurons utilizing catecholamine neurotransmitters. Over the past few years numerous investigations have focused on the regulatory mechanisms of this enzyme to determine if the synthesis of catecholamines could be manipulated in vivo. Because it has been speculated that the enzyme has limiting amounts of its cofactor in vivo we attempted in the present investigation to influence endogenous synthesis of dopamine by administering cofactor BH_4 and an analogue 6-methyltetrahydropterin ($6mPH_4$) which has been shown to cross the blood-brain barrier.

Methods: BH_4 and $6mPH_4$ were administered at a dose of 50 mg/kg and 100 mg/kg i.p. At different time periods the animals were given a sedative dose of barbital. Then the brain tissue in situ was flushed with saline (200 ml) by forcing through the left ventricle. The rat brain was then removed, and the striata dissected and frozen immediately. The tissues were analyzed several weeks later for the level of the particular pterin. Analysis was accomplished using HPLC and a reverse phase chromantics column (4.6 x 250 mm) ODS 5 micron. The flow rate was 1.3 ml/min using 5-10% methanol. All tissue samples were homogenized in acid, centrifuged and supernatant treated with I_2 to cause oxidation of BH_4 or $6mPH_4$. All solutions were then passed successively over a cation exchange column (Dowex 50 W-x4) and the anion exchange column (Dowex Ag 1-x2). The final acid eluate was lypholized overnight. Samples were then reconstituted in water and then analyzed by HPLC with a fluoresence detection 350/445.

The determination of dopamine and its metabolite DOPAC was accomplished using HPLC in combination with an electrochemical detector. The tissue samples were prepared by homogenization in 0.1N HAc and then centrifuged at 18,000 rpm for 20 min. The supernatants were decanted and an aliquot was then mixed with alumina, metabisulfide and 1M Tris in order to effect extraction of DA and DOPAC onto the alumina. The alumina was rinsed and then back extracted with 0.2 N HAc. The extract was analyzed directly by HPLC for DOPAC or diluted 1:5 before determining DA levels. The eluting solvent was phosphate buffered at pH 4.5 and containing EDTA and heptane sulfonic acid.

Major Findings: BH_4 administration at a dose of 100 mg/kg i.p. had no apparent effect on endogenous striatal levels of BH_4. However, $6mPH_4$ administration at a dose of 100 mg/kg i.p. resulted in significant levels of this compound occurring in striatal tissue. In addition the levels of striatal $6mPH_4$ remained elevated for over 150 min following the initial injection. We also analyzed these striatal samples for dopamine and its metabolite DOPAC. Following $6mPH_4$ injection there was only a small change in basal dopamine or DOPAC levels. A co-administration of $6mPH_4$ and tyrosine also had little or no effect on dopamine or DOPAC.

Significance to Biomedical Research: Since it had been speculated that tyrosine hydroxylase (TH) in vivo was subsaturated with respect to cofactor it was felt that this aspect of TH regulation could be conveniently manipulated. An ability for intervention of dopamine synthesis rates would have profound implications for application of this technique in disorders where dopamine neurotransmission was involved. However, the results from the present investigation suggest that an approach based on cofactor administration was ineffective under normal situations.

Proposed Course of Project: The possibility of increasing brain content of reduced cofactor has important implications for modifying neurotransmitter metabolism. Since tetrahydrobiopterin appears to only poorly cross the blood-brain barrier our efforts will be devoted toward obtaining and testing more lipophilic derivatives which are also catalytically active.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 03523-02 HE

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Administration of Tetrahydrobiopterin to Parkinson's Patients

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Leonard Miller	Guest Worker	HE NHLBI
OTHER:	Peter Lewitt	Clin. Assoc.	ET NINCDS
	Walter Lovenberg	Chief, Sect. on Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Degeneration of the nigral-striatal dopaminergic pathway is a major characteristic of patients with Parkinson's disease. Presently the etiology of this progressive degenerative process is unknown. However, the symptoms of this disorder can apparently be remedied by increasing neurotransmitter levels in the surviving neurons. This is accomplished by administering high levels of the precursor L-Dopa. The present investigation is also an attempt to increase endogenous dopamine neurotransmission by increasing the activity of the rate-limiting enzyme tyrosine hydroxylase. Hopefully this can be accomplished by raising the in vivo tissue levels of tetrahydrobiopterin (BH4). Our approach was to administer large doses of BH4 (2.0, 5.0 and 10.0 mg/kg) to patients while monitoring physiological responses as indicated in the protocol. Concurrent with this, CSF biopterin levels, DOPAC and HVA will be determined where possible. Our initial results with BH4 administration to Parkinson's patients revealed no significant effect on their normal motor functions, while their CSF level of BH4 was increased 4-5 fold.

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Objectives: Degeneration of the dopaminergic nigral-striatal pathway has been one of the major neurological characteristics of Parkinson's disease. Since this important observation many years ago clinicians have attempted to remedy this neuronal deficit by administering large doses of L-DOPA which is subsequently converted in vivo to dopamine. Recently we have taken the approach that dopamine synthesis can also be effected by increasing the activity of tyrosine hydroxylase (TH), the rate limiting enzyme in dopamine synthesis. It was felt that this could be accomplished since other investigators have speculated that TH is undersaturated with respect to its cofactor tetrahydrobiopterin (BH_4). Thus administration of large doses of BH_4 if it crossed the blood-brain barrier and accumulated sufficiently in dopaminergic terminals could possibly result in increased dopamine synthesis.

Methods: BH_4 will be obtained commercially in chemically pure form. Purity and freedom from bacterial contamination will be confirmed by the Pharmaceutical Development Section, NIH, who will be responsible for the preparation of this compound for its use in these studies. The BH_4 will be dissolved in an appropriate medium to insure its stability in solution for administration. Previous experience of human administration of BH_4 has involved the use of between 2.5 to 7.5 mg/kg body weight.

A patient will be hospitalized and observed for two days prior to administration of tetrahydrobiopterin. During that time, antiparkinsonian medications will be withheld. If the patient finds the absence of medication to be sufficiently distressing by the end of the second day, he or she may choose to reinstate the previous therapy, and tetrahydrobiopterin will not be administered.

We plan to administer 2.5 mg/kg in intravenous bolus form over five minutes, using an indwelling venous catheter. On succeeding days, doses of 5.0 mg/kg and 10.0 mg/kg will also be given in bolus form. During BH_4 administration, patients will be monitored for any change in heart rate, blood pressure, or neurological status. Evidence of any clinical improvement will be sought during the period following the administration of BH_4. Clinical features of parkinsonism will be evaluated by an observer in attendence, and video-tape records of changes in functional state will be recorded. In addition, motor function will be assessed with a battery of physiological tests, as described in Protocol 78-N-09, "Motor functioning patients with neurological diseases".

The patients will be tested for any evidence of toxicity by electrocardiogram, and blood evaluations including complete blood count and SMAC.

Major Findings: The drug was administered as in the protocol over a 3 day period with increasing successive doses of 2.5, 5.0 and 10.0 mg/kg i.v. The patients as monitored visually by Dr. Peter LeWitt showed no significant changes following drug treatment. CSF, BH_4 levels, however, had increased 4-5 fold.

Proposed Course of Project: The present results while initially unsuccessful should be pursued with a larger number of patients. Furthermore, patients in the early stages of the disorder might obtain more beneficial results from BH_4 administration than older patients whose state of degeneration is more profound. Such studies are planned.

Significance to Biomedical Research: Although our initial trial resulted in no significant clinical improvement, this study represents the first attempt to administer the hydroxylase cofactor and quantitatively measure its penetration into the CSF in parkinsonian patients. It should be noted that similar studies were done in two other countries, and a positive neurological response obtained.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03524-01 HE

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Protein Phosphorylation During the Development and Maintenance of Hypertension

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Paul Velletri	NIH Post-Doctoral Fellow	HE NHLBI
Other:	Walter Lovenberg	Chief, Sect.Biochem.Pharmacol.	HE NHLBI
	Judith Juskevich	Staff Fellow	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Post-translational modification of endogenous proteins by the introduction of phosphate groups occurs in peripheral cardiovascular tissues and in cardiovascular centers of the brain stem. The reaction is catalyzed by protein kinase, which can be activated by cAMP and cGMP, both of which are thought to be involved in blood pressure control. Protein kinase was characterized kinetically in brain stem, aorta and heart, and saturating conditions were established for the determination of Vmax. During the development of hypertension of spontaneous origin, there was no apparent difference in cyclic nucleotide-independent and cyclic nucleotide-dependent protein kinase activity in brain stem, aorta or heart of the spontaneously hypertensive rat (SHR) when compared to age-matched normotensive Wistar-Kyoto (NT-WKY) controls. However, significant age-related decreases in total kinase activity were observed in the aortas of SHR. These decreases were not observed in any other tissue studied. The significance of the drop in aortic kinase activity in SHR in relationship to blood pressure control is not understood at this time. Our laboratory is presently engaged in studying changes in endogenous substrate availability for protein kinase in the above tissues of the SHR.

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PHS-6040
(Rev. 2-81)

Objectives

There is considerable evidence suggesting that the cyclic nucleotides are involved post-synaptically in the regulation of blood pressure in peripheral cardiovascular tissue and in the cardiovascular centers of the brain stem (Hamet et al., Adv. Cyc. Nuc. Res. 12: 11, 1980; Walland, Naunyn-Schmiedeberg's Arch. Pharmacol. 290: 419, 1975). The primary macromolecular target of the cyclic nucleotides seems to be protein kinase, the enzyme that catalyzes the phosphorylation of endogenous tissue proteins (Cohen, Nature 296: 613, 1982).

Due to the presumed role for cyclic nucleotides in blood pressure regulation, a few investigators have attempted to study the relationship of cAMP-dependent protein kinase activity in cardiovascular tissues to the hypertensive disease process. Previous reports suggest conflicting results, with some describing an increase (Sands et al., Blood Vessels 13: 361, 1976) and others a decrease (Bhalla et al., Hypertension 2: 207, 1980; Coquil and Hamet, Proc. Soc. Exp. Biol. Med. 164: 569, 1980) in aortic cAMP-dependent protein kinase activity in SHR when compared to age-matched NT-WKY controls. However, the measurement of protein kinase activity in these studies does not appear to have been determined employing saturating enzyme kinetic analysis, which is an accurate index of enzyme concentration. The purpose of the present study was to measure the activity of cAMP- and cGMP-dependent protein kinase in the brain stem, aorta and heart of the SHR and NT-WKY at three different ages in an attempt to determine if basal or cyclic nucleotide-dependent protein kinase activity might change during the development of hypertension of spontaneous origin.

Methods

Wistar-Kyoto SHR and NT-WKY from the NIH colonies were used in all studies. Blood pressures were measured at 4, 8 and 16 weeks of age using standard plethysmographic techniques (Okamoto and Aoki, Jap. Circ. J. 27: 282, 1963). Animals were also sacrificed at 4, 8 and 16 weeks of age for the determination of protein kinase activity in brain stem, aorta and heart.

Rats were sacrificed by decapitation. The brain stem was isolated from the upper brain at the level of the tegmentum, and included the medulla, pons and the superior and inferior colliculi. The aorta, from the aortic arch to the abdominal bifurcation, was dissected free of surrounding musculature and connective tissue. The heart was freed of residual vasculature and connective tissue. All tissues were rinsed in isotonic saline to remove adhering blood, and frozen on dry ice for up to 2 hours prior to homogenization.

Homogenization was carried out in a teflon-glass homogenizer. All tissues were homogenized in 5 vols. 20 mM Tris HCl, pH 7.4. All three homogenates were centrifuged at 1000 g for 10 min at 4°C and the crude supernatant was kept for enzyme and protein assays.

Protein kinase activity was determined in a final incubation volume of 200 µl containing 20 mM Tris HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM theophylline, 200 µg histone IIA, 75-100 µg tissue protein, 500 µM [γ^{32}P] ATP (50×10^3 dpm/nmol) and either 100 µM cAMP or cGMP, when added. Reactions were carried out at 30°C for 5 min. The enzymatically catalyzed reaction was terminated by the addition of 1 ml 20% trichloroacetic acid to the reaction mixture and the reaction tubes were placed on ice. The reaction mixture was then filtered through Type HA Millipore filters and residual reaction mixture was washed from the glass tubes

twice with 4 ml 10% trichloroacetic acid. Filters were transferred to glass scintillation vials to which 10 ml Filtron-X counting fluid was added. Vials were counted for 5 min. Activity was expressed as pmol [γ^{32}P] phosphate incorporated into histone IIA/min x mg protein.

The conditions described for the protein kinase assay were optimized for the measurement of maximal protein kinase activity (Vmax). Experiments were performed to insure: the linearity of product formed as a function of time; the linearity of enzyme velocity as a function of tissue protein concentration; and saturating conditions as a function of ATP, histone IIA, cAMP, and $MgCl_2$ concentrations.

Major Findings

Saturating conditions for protein kinase can be achieved in brain stem, aorta and heart if the following reaction conditions are met: reagent concentrations - 500 µM ATP, 10 mM $MgCl_2$, 1 mg/ml histone IIA, 100 uM cAMP or cGMP; tissue protein concentration - 100 µg/200 ul for brain stem and aorta, and 75 µg/200 µl for heart; incubation time - 5 min at 30°C and pH 7.4. These conditions insure that an accurate estimate of Vmax, and therefore enzyme concentration, can be obtained. (Vmax = k_2 x enzyme concentration. See discussion in Morris, A Biologist's Physical Chemistry. Reading, Addison-Wesley Pub. Co., 1974).

Blood pressures of SHR and NT-WKY were identical at 4 weeks of age, but by 8 weeks of age SHR had blood pressures of 161 ± 2 mm Hg as compared to 131 ± 3 mm Hg in NT-WKY. At 16 weeks of age, SHR had blood pressures of 201 ± 6 mm Hg compared to 125 ± 8 mm Hg in NT-WKY.

No difference in basal or cyclic nucleotide-dependent protein kinase activity was observed in brain stem, aorta or heart at 4, 8 or 16 weeks of age when SHR were compared to their age-matched NT-WKY. Generally, basal and cyclic nucleotide-dependent protein kinase activity appeared to be higher in all tissues of the 4 week-old animals when compared to values from the 8 week-old or 16 week-old animals.

A noticeable trend towards an age-related decrease in basal and cyclic nucleotide-dependent protein kinase activity in aorta and heart of SHR and NT-WKY could be distinguished. This age-related drop in kinase activity appeared to be most pronounced in the aorta of SHR. In contrast, the kinase activity in the aorta of NT-WKY did not change significantly with age. The brain stem and heart from both SHR and NT-WKY also did not show a significant drop in kinase activity with age. Hence, although aorta and heart appeared to display trends towards a drop in kinase activity over time, only the aorta of the SHR had a trend that was statistically significant.

Significance to Biomedical Research and Institute Programs

As a large disparity of results has appeared previously in the literature concerning changes in protein kinase activity in hypertension, a major achievement of the present research is its attempt at resolving the present controversy. Our interest in resolving the controversy concerning protein kinase in hypertension has led to the measurement of kinase activity under saturating conditions. Hence, our enzyme activities represent zero-order kinetics. As previous investigations were conducted using conditions that were not saturating, it seems likely that the enzyme activities reported in those papers represented mixed order

kinetics. Under such circumstances it would be difficult to determine the nature of the enzymatic change that was occurring during the hypertensive disease process. We feel that one of our most important contributions in this field to date has been our effort at controlling carefully the conditions of the enzymatic reaction. Such precision is especially important when applying biochemical techniques to hypertension research, as this area of study has traditionally suffered from a dearth of biochemical investigations.

The results of the present study are in disagreement with previously published reports. We have been unable to detect any differences in cyclic nucleotide-independent and cyclic nucleotide-dependent protein kinase activity between SHR and NT-WKY in any tissue or age group studied. However, there was a unique age-related drop in total kinase activity in the aorta of SHR, a finding that is in agreement with one report (Coquil and Hamet, Proc. Soc. Exp. Biol. Med. 164: 569, 1980). The biological significance of this drop in kinase activity in relationship to blood pressure regulation is not known at this time.

There are at least a few factors that could account for the discrepancy between our results and the results that have been reported previously. First of all, it must be emphasized again that our study was conducted under saturating enzymatic reaction conditions, which allow for the measurement of Vmax. Hence, it would appear safe to conclude from our data that the Vmax, and therefore the enzyme concentration, for basal and cyclic nucleotide-dependent protein kinase does not differ between SHR and NT-WKY in any tissue at any particular age group. Secondly, it is possible that because our methodology for subcellular fractionation was different from that of previous reports, different results could be obtained. However, our 1000 g crude supernatant contained all the subcellular components (i.e., microsomes and soluble fractions) that were assayed in other studies, and therefore one would expect that our results should not differ profoundly on the basis of tissue preparation alone. Therefore, the kinetic sources of the changes described in earlier studies appear puzzling, although differences in the affinity of the catalytic subunit for exogenous protein substrate and ATP or differences in the affinity of binding of the cyclic nucleotides to the regulatory subunit must be considered.

Neither the present study nor any studies appearing previously in the literature can address the problem whether potential observed changes in protein phosphorylation could be the cause or result of the hypertensive disease process. Investigations have also been hindered by the lack of experiments that deal with endogenous protein substrate availability during the development and maintenance of hypertension. As no changes in phosphoprotein phosphatase have yet been observed in hypertension (Bhalla et al., Hypertension 2: 207, 1980), and, as it is the contention of the present study that levels of cyclic nucleotide-dependent protein kinase do not differ between SHR and NT-WKY, it seems reasonable to assume that if changes in protein phosphorylation do in fact occur in the hypertensive disease state, then they might well occur in endogenous protein substrate availability.

Proposed Course of Project

The following experiments are planned to assess more completely the role of protein phosphorylation in the development and maintenance of hypertension:

1. Crude supernatants derived from brain stem, aorta and heart at 4, 8 and 16 weeks of age will be assayed to study endogenous protein

Phosphorylation in SHR and NT-WKY. The phosphorylation of endogenous proteins will be studied employing denaturing slab gel electrophoretic techniques.

2. If effects on endogenous substrate availability are noted during the course of the hypertensive disease process, treatment with antihypertensive agents will be instituted to ascertain what effects, if any, such treatment has on phosphorylation. Agents to be used will include sympatholytics, diuretics and directly-acting vasodilators.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03525-01 HE

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Dipeptidyl Carboxypeptidase Activity in Rat Brain and Adrenal Medulla

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Other:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The activity of dipeptidyl carboxypeptidase (DCP) was studied in rat brain stem using the substrate [3H]-benzoyl-phenylalanyl-alanyl-proline. Assays were performed using first-order enzyme reaction kinetics. The highest specific activity of DCP was found in the 49,500 g particulate fraction, with less activity in the 1000 g pellet and virtually no activity in the 49,500 g supernatant. This enzyme was present in both intact crude synaptosomal preparations and in the membrane-fraction of lysed synaptosomes, providing strong evidence that DCP is a membrane-bound enzyme. Depolarizing conditions did not alter enzyme activity. DCP activity was chloride ion sensitive and could be inhibited by captopril and EDTA. When the crude synaptosomal preparation was purified further, the highest specific activity of DCP was found in the enriched synaptosomal fraction. Although the endogenous substrate(s) for DCP in brain stem could be angiotensin I or bradykinin, it is also possible that this enzyme is involved in the synthesis/degradation of the enkephalins. Further characterization of the biochemical properties and physiological regulation of DCP is presently being undertaken using the adrenal medulla, a tissue rich in the enkephalins, as a model system.

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Objectives: The presence in brain tissue of a dipeptidyl carboxypeptidase similar to angiotensin-converting enzyme (DCP; ACE; E.C. 3.4.15.1) has been reported by a number of investigators (Cushman and Cheung, Biochim. Biophys. Acta 250: 261, 1971; Yang and Neff, J. Neurochem. 19: 2443, 1972; Poth et al., J. Neurochem. 25: 83, 1975). However, the precise role of brain DCP, which in the periphery is responsibe for the conversion of angiotensin I to angiotension II and for the inactivation of bradykinin (Soffer, Ann. Rev. Biochem. 45: 73, 1976), is unknown. Recent reports suggest that brain neurons do not synthesize angiotensin (Meyer et al., J. Neurochem. 38: 816, 1982) and the actual presence of a brain neuronal renin-angiotensin system is highly controversial (Reid, Fed. Proc. 38: 2255, 1979; Phillips et al., Fed. Proc. 38: 2260, 1979). However, there are studies that indicate that all components of a renin-angiotensin system, including DCP (ACE), are present in certain cell lines of neuroblastoma cells (Okamura et al., Proc. Natl. Acad. Sci. 78: 6940, 1981). It is possible that DCP activity in brain neurons may be responsible for the inactivation of met-enkephalin (Benuck and Marks, Biochem. Biophys. Res. Comm. 88: 215, 1979; Arregui et al., Eur. J. Pharmacol. 59: 141, 1979). It appears obvious that the precise role of DCP in neuronal tissue remains unclear.

Some investigators have recently reported changes in DCP (ACE) activity in the brains of spontaneously hypertensive rats (SHR) when compared to age-matched normotensive Wistar-Kyoto (NT-WKY) rats (Polsky-Cynkin et al., Proc. Soc. Exp. Biol. Med. 164: 242, 1980; Mendelsohn et al., Clin. Exp. Pharmacol. Physiol. 7: 551, 1980). Furthermore, Chevillard and Saavedra (J. Neurochem. 38: 281, 1982) have shown a unique distribution of DCP (ACE) in specific areas of the rat brain stem, which is known to contain a number of cardiovascular control centers (Juskevich and Lovenberg, Biochem. Act. Horm. 8: 117, 1981). However, to date there have been no studies precisely defining the subcellular distribution of DCP in any brain region. The purpose of the preliminary studies described in this annual report was to determine if DCP is present in brain synaptosomes and to proceed to define the biochemical and physiological role of this enzyme in neuronal tissue. Brain stem was chosen as a preliminary model tissue to study due to work that has been reported previously (Chevillard and Saavedra, J. Neurochem. 38: 281, 1982). However, a peripheral model system employing the adrenal medulla is presently being considered due to the ease by which this tissue can be manipulated pharmacologically and physiologically.

Methods: Male Sprague Dawley rats were decapitated and the brain stem at the level of the tegmentum was dissected free from upper brain regions, and included the medulla, pons and superior and inferior colliculi. Brain stems were washed in 0.32 M sucrose to remove all adhering blood. This step is essential as plasma contains high concentrations of DCP (ACE).

For the initial subcellular distribution studies on DCP, brain stems were homogenized in 5 vols. of ice-cold 20 mM Tris-Acetate, pH 7.4, a buffer tonicity that will presumably destroy all subcellular vesicles. In these studies, brain stem homogenates were centrifuged at 1,000 g for 10 min (4°C). The 1,000 g pellet was resuspended in 50% of the original homogenate volume using 20 mM Tris-Acetate and saved for assay. The 1000 g supernatant was centrifuged at 49,500 g for 30 min (4°C) and the pellet was resuspended in 15% of the original homogenate volume using 20 mM Tris-Acetate, pH 7.4, and saved for assay. The 49,500 g supernatant was also saved for assay.

For all subsequent studies employing intact or lysed synaptosomal preparations, brain stems were homogenized in 5- 10 vols. of ice-cold 0.32 M sucrose in a Thomas Type A teflon-glass homogenizer (clearance: 0.10-0.15 mm). Synaptosomes were prepared by methods described previously in the literature (Gray and Whittaker, J. Anat. 96: 79, 1962; Jones and Matus, Biochim. Biophys. Acta 356: 276, 1974; Burke and Delorenzo, Brain Res. 236: 393, 1982). For synaptosomal studies, the 1,000 g pellet was discarded and the 1,000 g supernatant was centrifuged at 10,000 g for 20 min (4°C). In those studies using intact crude synaptosomes, the 10,000 g pellet was resuspended in 12.5% of the original homogenate volume using 0.32 M sucrose. If a discontinuous sucrose density gradient was to be run, 1 ml of the resuspended 10,000 g pellet (25% of the original homogenate volume) was layered on top of a discontinuous gradient composed of 5 ml of 1.2 M and 5 ml of 0.8 M sucrose. The gradient was centrifuged in a swinging bucket rotor head at 81,500 g for 120 min (4°C). The mitochondrial pellet (at the bottom of 1.2 M sucrose) was resuspended in 7.5% of the original volume with 0.32 M sucrose. The myelin band (at the 0.32 M/0.8 M interface) and the synaptosomal band (at the 0.8 M/1.2 M interface) were diluted 1 : 2 with 20 mM Tris-Acetate and centrifuged at 100,000 g for 30 min (4°C) and then resuspended in 6.25% of the original homogenate volume using 0.32 M sucrose. In those studies in which crude synaptosomes were lysed, the 10,000 g pellet was resuspended in 6.25% of the original homogenate volume with 20 mM Tris-Acetate, pH 7.4, and allowed to sit on ice for 30 min. This suspension was then centrifuged at 49,500 g for 30 min (4°C) and the pellet resuspended in 12.5% of the original volume with 20 mM Tris-Acetate, pH 7.4.

The assay system was modified from the method of Ryan et al. (Tiss. Cell. 10: 555, 1978), which uses [^{3}H]-benzoyl-phenylalanyl-alanyl-proline as a substrate. Final incubation volumes were 200 ul. Assay buffers were composed of one of the following: (1) 20 mM Tris-Acetate, pH 7.4, with 0.1 M NaCl; or (2) 125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM D-glucose and 50 mM Tris-Acetate, pH 7.4. Assay buffer 1 was used for lysed synaptosomes and assay buffer 2 was used for intact synaptosomes. When depolarizing conditions for the intact synaptosomes were desired, both 65 mM NaCl and 65 mM KCl were employed. Final osmolality was always calculated to be 326 mosM. In most cases, 100 ug tissue protein per assay tube were used. However, under certain conditions, 25 ug tissue protein were assayed. When added, captopril concentrations were 10 uM. [^{3}H]-benzoyl-phenylalanyl-alanyl-proline was used as a substrate for DCP. Reaction kinetics in these studies were first-order with respect to substrate concentration. 200,000-400,000 cpm (9-18 pmol) of substrate were used per assay tube.

The reaction was initiated by the addition of substrate, or when using intact synaptosomes, by the addition of tissue. Reaction time varied from 5-20 min and was carried out at 37°C. The reaction was stopped by the addition of 1 ml of 0.1 N HCl. Product ([^{3}H]-benzoyl-phenylalanine) was extracted by adding 1 ml of a toluene-based extraction-counting fluid (Ventrex Inc. Scintillation Cocktain 2). Samples were vortexed for 10 sec and then centrifuged in a swinging bucket rotor head at 1,000 g for 20 min. 500 ul of the upper organic phase were placed in microvials and counted in 20 ml scintillation vials for 5 min. The partition coefficient for benzoyl-phenylalanine is 1. Hence, a correction factor of 4 is employed for both partitioning between aqueous and organic phases and for the sampling of half the volume in which product formed is dispersed. Blank values were approximately 1-2% of the total counts. As the reaction was first-order, units of enzyme activity were expressed as fractional conversion (cpm product/cpm total substrate) per µg protein and per total reaction time.

The fractional conversion of substrate to product per 10 min was linear with tissue protein up to 100-125 ?g protein. Hence, DCP activity was expressed as fractional conversion per ?g protein per total reaction time (10-15 min).

First-order kinetic conditions simulate the presumed endogenous concentrations of substrates for DCP, which are on the order of 0.05 nM (Backle, Handbook Exp. Pharmacol. 37: 41, 1974). The substrate concentrations used in the present in vitro assays were approximately 1000-fold higher than in vivo endogenous substrate concentrations, but were still first-order. First-order kinetic conditions also allow for the most sensitive and accurate detection of enzyme activity, as the specific radioactivity of substrate is maximal. The fractional conversion of substrate (product/substrate ratio) is directly proportional to the Vmax of the enzyme and indirectly proportional to the Km (White et al., Principles of Biochemistry, 1973). Other advantages of using first-order kinetics for enzyme analysis include the fact that the order with regard to substrate of the kinetic reaction is known. However, the first-order conditions do not replace the information that can be obtained using higher substrate concentrations, and classical Michaelis-Menten kinetic analysis will be performed at a future date.

Major Findings: Eighty-eight percent of the total DCP activity in brain stem was localized in the 1,000 g pellet, which is composed of crude cellular debris, nuclei and very large membranous particles. Of the remaining 12% of DCP activity, over 75% of DCP activity was in the 49,500 g pellet. The soluble component of the subcellular fractions possessed between 2-3% of the total crude homogenate enzyme activity. Over 90% of DCP activity in all fractions studies was inhibited by captopril. The highest specific activity of DCP was localized in the 49,500 g pellet. Total homogenate specific activity was 0.576 per 1000 ?g protein per 15 min, as versus 0.924 in the 49,500 g pellet and 0.720 in the 1000 g pellet.

The crude mitochondrial pellet (10,000 g) was examined further for DCP activity, as this fraction is contained within the 49,500 g pellet and also is rich in synaptosomes. Both the intact and lysed 10,000 g pellets contained equivalent DCP activity (within 10% of each other), and the enzyme activity was inhibited by captopril. These results indicate that DCP is a membrane-bound enzyme, as both intact synaptosomes and synaptosomal membranes devoid of synaptoplasm contained equivalent enzyme activity. When a discontinuous sucrose density gradient was run on the resuspended 10,000 g pellet, it was noted that only synaptosomes contained an elevated specific activity in comparison to the crude synaptosomal preparation. Enriched synaptosomes contained over 2-fold the DCP activity that was present in the crude synaptosomal preparation.

Although DCP activity showed chloride sensitivity, about 50% of total activity was still present in the absence of added NaCl, either due to a lack of chloride dependence or incomplete removal of chloride ion from the tissue preparation. Depolarizing conditions did not dramatically affect DCP activity in crude synaptosomes.

Significance to Biomedical Research and Institute Programs: The results obtained thus far from preliminary studies demonstrate the presence of DCP of high specific activity in rat brain stem synaptosomes. The enzyme appears ACE-like in that it is chloride sensitive and inhibited by captopril. However, as the presence of angiotensin I has not been conclusively demonstrated in neurons (Meyer et

al., J. Neurochem. 38: 816, 1982), the question as to the role of DCP in brain neurons remains unanswered. The fact that DCP is present in synaptosomes may act as a processing enzyme for the synthesis of small molecular weight peptides in nerve terminals. We know of no other reports in the literature that have localized DCP to synaptosomes.

Carboxypeptidase activity may be necessary for the synthesis of met- and leu-enkephalin from larger precursors (Hook et al., Nature 295: 341, 1982). The exact identity of the enzyme involved in such peptide processing is at present unknown. Enkephalins appear to be present in adrenal chromaffin granules (Viveros et al., Adv. Biochem. Psychopharmacol. 22: 191, 1980), and may actually exist as co-transmitters in noradrenergic vesicles (Wilson et al., Nature 288: 707, 1980). Studies performed in a number of laboratories (Jones et al., Proc. Natl. Acad. Sci. 79: 2023, 1982) have defined the amino acid sequence for enkephalin precursors, but not the metabolic reactions that are involved in peptide processing from larger to smaller molecular weight peptides. Hook et al. (Nature 295: 341, 1982) are among the first investigators to study possible enzymes involved in enkephalin processing.

The adrenal medulla appears to be an excellent and relatively easy model for studying enkephalin processing (Viveros et al., Adv. Biochem. Psychopharmacol. 22: 191, 1980) and may possess high specific activity of DCP, as does the brain stem. Indeed, it may be possible that those tissues high in enkephalins are also high in DCP activity. Striatum, for instance, which is believed to possess met-enkephalinergic neurons (Hong et al., Neuropharmacol. 16: 451, 1977), also has among the highest reported specific enzyme activity for DCP (Yang and Neff, J. Neurochem. 19: 2443, 1972). If, as some speculate (Wilson et al., Nature 288: 707, 1980), the enkephalins co-exist with catecholamines in nerve terminals, the relationship of catecholamine and enkephalin metabolism would be an interesting and valuable area of pursuit. DCP would be a particularly interesting enzyme to study in that it appears to be specifically inhibited by the antihypertensive agent captopril (Ondetti et al., Science 196: 441, 1977).

Eventually, the preliminary studies reported here may develop into a complete exploration of DCP in blood pressure regulation and also lead to a study of the possibly interrelated roles of catecholamines and opiod-like compounds in blood pressure homeostasis. It is essential to consider that crucial to these investigations is the hypothesis that DCP acts on endogenous substrates other than angiotensin I and bradykinin.

Proposed Course of Project: The following areas of research will be pursued over the remaining year in order to clarify further the biochemical identification and physiological regulation of DCP:

1. The adrenal medulla will be used as a model system to study DCP. Carboxypeptidase activity and the presence of enkephalins have been reported in this tissue. The adrenal medulla is easily accessible and isolated from surrounding tissue, and can be subjected to a variety of physiological and pharmaco logical manipulations in an attempt to define regulatory mechanisms for DCP.

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2. The possibility that enkephalins are *in vivo* products of DCP activity will also be explored using peptide precursor studies.

3. The role of DCP in discrete tissues (such as brain stem and renal medulla) during the development of hypertension will be studied in the spontaneously hypertensive rat. As a corollary to this work, the actions of captopril administered *in vivo* on these tissues will be studied.

4. Michaelis-Menten kinetic analysis will be employed when necessary to determine if observed catalytic changes are due to alterations in Km or Vmax.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03526-01 HE

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Effects of Adenine Nucleotides on 3H Dopamine Binding in Rat Brain Striatal Tissue

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	William A. Wolf	Guest Worker	HE NHLBI

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None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The biochemical regulation of the dopamine receptor was studied in vitro. Adenosine triphosphate (ATP) and adenylyl imidodiphosphate (AMPPNP), a non-hydrolyzable analogue of ATP, both enhance the binding of 3H dopamine to rat brain striatal tissue. Adenylate cyclase activity did not appear to be involved in this effect nor does Na+-K+ ATPase activity appear to be involved. Studies with sodium orthovanadate, however, suggest that a Ca+2 or Mg+2 ATPase may be involved.

PHS-6040
(Rev. 2-81)

Objectives: Intense investigation in the field of neurotransmitter receptors has led to the discovery and pharmacological characterization of many types of binding sites for endogenous neurotransmitters and other compounds. A clear understanding of the biochemical mechanisms of transduction of the chemically transmitted signal at the receptor level and the regulation of these receptor-mediated events, however, has been more elusive. Since these phenomena are the basis for synaptic transmission it seems reasonable to attempt to delineate the mechanisms involved. The dopamine receptor system was chosen because there is a vast field of research, albeit somewhat confusing, from which to draw inferences and formulate theories as to the biochemical processes which dopamine affects via the receptor and, in turn, the biochemical mechanisms which regulate the activity of the dopamine receptor system.

Methods: Rat brain striatal tissue was prepared and ^{3}H dopamine receptor binding was assayed in a manner similar to Burt et al. (Mol. Pharmacol. 12:800, 1976). The effects of various ions (Ca^{+2}, Na^{+}, K^{+}, Mg^{+2}) on the binding of dopamine at equilibrium was studied. The effects of adding ATP and AMPPNP, a non-hydrolyzable analogue of ATP, were studied under various conditions to determine if a cAMP dependent or Ca^{+2}-Calmodulin dependent phosphorylation mechanism was involved in the regulation of the dopamine receptor. In addition the effects of ouabain and sodium orthovanadate were studied in order to determine if dopamine binding was involved in an ATPase reaction mechanism (e.g. Na^{+}-K^{+} ATPase, Ca^{+2}-ATPase, Mg^{+2}-ATPase).

Major Findings: Two binding sites for ^{3}H dopamine were found in the rat brain striatal tissue which had markedly different affinities. The low affinity binding site had an apparent K_D of approximately 6 μM while the high affinity binding site had an apparent K_D of approximately 20 nM. The physiological significance of the low affinity site only remains a question since it was present in boiled tissue preparations, however, both sites exhibited a typical "dopaminergic" profile (i.e. dopamine and dopamine agonists were more potent in displacing ^{3}H dopamine than other catecholamines and other compounds). The binding of ^{3}H dopamine to both sites was enhanced approximately 2 fold in the presence of Ca^{+2} ions. The other ions investigated did not appear to have a significant effect on dopamine binding to either site. The addition of ATP or AMPPNP enhanced the binding of dopamine to both sites several fold over the binding seen with Ca^{+2} alone. Mg^{+2} was required for the ATP and AMPPNP effects. The effect seen with ATP was somewhat different from that seen with AMPPNP suggesting the possible involvement of not only an allosteric effect (i.e. the effect seen using AMPPNP), but a phosphorylation mechanism (i.e. the effect seen using ATP) as well. Neither the addition of cAMP with or without exogenously added cAMP dependent kinase nor the addition of Ca^{+2} plus calmodulin affected the ATP enhanced ^{3}H dopamine binding. Various known activators of adenylate cyclase (NaF, forskolin, GMPPNP, cholera toxin) and ouabain had no effect, however, sodium orthovanadate was found to inhibit the ATP or AMPPNP enhanced dopamine binding with an IC_{50} of approximately 10-20 μM. These results suggested that although neither adenylate cyclase nor a Na^{+}-K^{+} ATPase was involved another ATPase reaction mechanism (e.g. Ca^{+2}/Mg^{+2}-ATPase) might be. Further kinetic studies were performed on the vanadate inhibition in order to characterize it and draw possible parallels between the effects of vanadate on the ATP enhanced dopamine binding and documented characteristics of vanadate on Ca^{+2}/Mg^{+2} ATPase activity.

In addition preliminary experiments have been performed on the effects of dopamine on ^{3}H-AMPPNP binding under the same conditions in which AMPPNP enhances dopamine

binding. These experiments have shown that the presence of added dopamine (100 nM-1 uM) alters the binding kinetics of ^{3}H-AMPPNP to rat brain striatal tissues further supporting the suggestion that dopamine may alter ATPase activity.

Significance: The present experiments suggest that the interaction of dopamine with the observed binding sites is closely related to a reaction mechanism which involves ATP but which is not the dopamine sensitive adenylate cyclase. Further analysis suggests that the binding of dopamine to these sites may reflect the activity of a Ca^{+2}/Mg^{+2}-ATPase. This may be a means of regulation of the dopamine receptor, or a novel neuronal uptake system for dopamine or may represent one of the mechanisms of action of dopamine receptor-mediated neurotransmission. This latter conclusion has some support from previous work in the field of ion translocating ATPases (see Antonelli de Gomez de Lima et al., Neurochem. Res. 6:969, 1981, and Undesser et al., Soc. Neurosci. Abs. 7:572, 1981). In light of the fact that dopamine has been proposed to act centrally and peripherally in the reflex regulation of blood pressure and heart rate and dopamine analogues have been studied as possible antihypertensive agents (see Cavero et al., JPET, 219:510, 1981 and refs. within) a further understanding of the biochemical mechanisms underlying these phenomena as has been attempted in the present experiments may facilitate the research and development of effective means of treating certain cardiovascular disorders.

Proposed Course of Project:

1. Determine further characteristics of the ATP enhanced dopamine binding (e.g. presynaptic or postsynaptic localization).

2. Fully characterize the effect of dopamine, dopamine agonists and antagonists on AMPPNP binding.

3. Determine the effects of dopamine, dopamine agonists and antagonists, on the kinetics of ATPase activity.

4. Determine the effects of dopamine, dopamine agonists and antagonists, on Ca^{+2} binding and uptake.

Publications:

None to date

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03527-01 HE

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

In Vitro Study of the Regulatory Features of Rat Striatal Tyrosine Hydroxylase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Leonard Miller	Guest Worker	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharm.	HE NHLBI

COOPERATING UNITS (if any)

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LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

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0.5	0.5	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The present investigation analyzed rat striatal tyrosine hydroxylase in vitro at pH 7.2 using the natural cofactor 6(R) L-erythrotetrahydrobiopterin. Both control enzyme activity and activated enzyme were examined. The results showed that there are two different forms of the enzyme. One form has an apparent Km of 240 uM and upon phosphorylation is converted to 8 uM. The other form has a Km of 1.4 mM and appears unaffected by phosphorylation. In addition, enzyme activity was not linear for time during the first 10-15 min of incubation. This non-linearity was not apparent at low cofactor concentrations using enzyme activated by phosphorylation.

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Objectives: Since the initial observation that tyrosine hydroxylase (EC 1.14.15.2; tyrosine 3-monooxygenase) is the rate limiting enzyme in catecholamine biosynthesis there have been numerous investigations in an attempt to uncover the regulatory features which underlie the apparent limitation of synthesis rates in vivo. Initially end-product inhibiton by catecholamines was described. Further insight was obtained when it was shown that the enzyme itself could be activated reuslting in either a K_m, K_i, or V_{max} change. Additional studies have suggested that the activation is mediated by direct phosphorylation of the enzyme. Recently, some investigators have begun to examine TH at, or close to, physiological pH. It appears that at 7.0-7.4 there are marked differences in the levels of enzyme activity between control and activated enzyme. While all of these studies have provided increased understanding of TH regulation, most have used one of the pterin analogues of the natural cofactor such as 6-methyl- or 6,7 dimethyltetrahydropterin. To date, there have been no examinations of striatal TH using solely the natural cofactor (6R) L-erythrotetrahydrobiopterin. Therefore, the present study was undertaken to examine TH activity at pH 7.2 using the natural cofactor and, in addition, to investigate the efects of in vitro phosphorylation on enzyme kinetics.

Methods: (6-R)-L-erythrotetrahydrobiopterin preparation: Tetrahydrobiopterin as received from the laboratory of Dr. Schircks, Switzerland, consisted of the two 6-diastereoisomers, 6R and 6S forms, in an approximate ratio of 2.5:1, respectively. Since it has been shown that the 6R form is the naturally occurring cofactor, it was decided to separate this form from the mixture. This was accomplished using HPLC by passing 300 ug of the mixture in 30 ul H_2O over a (4.6 mm x 25 cm) column of Partisil SCX, 10 micron. The eluting solvent was 12.5 mM aqueous ammonia adjusted to pH 3.3 with acetic acid. The compounds were detected by a UV lamp set at 254 nm. The peaks indicating the natural form were collected, lyophilized overnight and used the next day. An HPLC analysis of the purified fraction showed only one peak (Fig. Ib). The concentration of cofactor was determined at O.D. of 264 in 0.1 N HCl and using a molar extinction coefficient of 16,000.

Tissue preparaton: Male Sprague-Dawley rats (220-300 grams) were killed by decapitation, striata removed and homogenized immediately in 4 volumes (ml/gr wet wgt.) of 0.5 M KP, pH 7.2 containing 0.2% Triton X-100. The homogenate was then spun at 18,000 RPM for 40 min and the supernatant used for analysis. In the initial series of experiments, endogenous catechols were removed by passing the supernatant over a 0.5 x 2 cm column of Dowex 50 W-X4. The eluate was then used directly or diluted. For the phosphorylation analysis, 400 ul of either controls or samples with phosphorylating mix were passed over a 1 x 30 cm column of Sephadex G-25 preequilibrated with 0.5 M KP, pH 7.2. The eluate was used directly for analysis.

Phosphorylating conditions: 200 ul of striatal supernatant was combined with 200 ul of H_2O containing ATP (2 mM), cAMP (0.4 mM), $MgCl_2$ (20 mM), theophylline (2 mM). Then 20 ul of protein kinase (200 ug) were added and the mix was incubated for 10 min at 30°C. The samples (400 ul) were then passed over a Sephadex G-25 column as described above to remove the phosphorylating mix from protein.

Tyrosine hydroxylase activity determination: Tyrosine hydroxylase activity was determined using a slight modification of the tritium release method which has been previously described. In brief, activity was measured by determining the amount of tritiated water formed following the conversion of tritiated tyrosine to DOPA. Labelled tyrosine was initiallly purified by first diluting with 5.0 ml H_2O, adjusting to pH 2.0 in HCl then passing over a 6 mm x 65 mm column of Dowex 50-X4. The column was rinsed with 10 ml H_2O then 30 ml 0.5 N HCl. The label was eluted with 40

ml 1N HCl. The sample was lyophilized to dryness and then brought up in ethanol:H_2O (1:3) and kept refrigerated until needed. On the day prior to the experiment, a small aliquot (150 -250 ul) of the purified batch was lyophilized overnight. Equal volumes of the following solutions were added to the ^3H-tyrosine: 0.5 mM tyrosine, 1 mM ferrous ammonium sulfate (made daily), 0.5 M KP; pH 7.2, 0.2 M ascorbic acid (made daily) and catalase (3.4×10^5 units/ml). Fifty microliters of this mixture was added to each test tube followed by 10 ul containing BH_4 in 0.01 NHCL. The reaction was initiated by adding 40 ul of treated supernatant, incubated for the indicated time periods at 37°C and then terminated by adding 400 ul 5% TCA. The tritiated water formed was separated from tritiated tyrosine and DOPA by a dual resin column of: (bottom layer) Dowex 50W-X4 (H^+) (0.65 x 2.5 cm), a thin layer of charcoal then Dowex Agl-X2 (acetate) (0.6 x 1.0 cm). The samples were rinsed onto the columns with 2 x 0.7 ml of water. Fifteen ml of ACS was added to the combined column eluates and then counted by standard technique in a liquid scintillation counter.

Major Findings: The present investigation using striatal tyrosine hydroxylase at pH 7.2 showed: (A) Nonlinearity of enzyme activity during the initial period of a time course analysis in control enzyme. This nonlinearity was no longer apparent following phosphorylation of the enzyme using only low levels of cofactor (4-200 uM). At higher cofactor levels (400-1500 uM) the nonlinearity during the early time period was still evident in those samples treated with phosphorylating conditions. (B) Double-reciprocal plots suggest the presence of at least three kinetically different forms of the enzyme with apparent K_m's of 1400, 240 and 8 uM, and V_{max}'s of 7.9, 2.4 and 0.4 pmoles/ugr protein/15 min, respectively. (C) Treatment of homogenates with phosphorylating conditions suggest the interconversion of two different forms, i.e. from 240 uM to 8 uM.

Significance to Biomedical Research: The present investigation has uncovered an extremely low K_m form of tyrosine hydorxylase (K_m = 8 uM). The significance of this finding remains to be established beyond the fact that it appears to be the activated form of the enzyme. The nonlinearity of enzyme activity with respect to time during the first 10-15 min of incubation is an important observation in terms of determining the proper amount of enzyme present in a tissue homogenate.

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PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Nutritional Factors and the Incidence of Stroke in Genetically Hypertensive Rats.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Walter Lovenberg	Chief, Sect. Biochem. Pharm.	HE NHLBI
OTHER:	Dr. Yukio Yamori	Guest Worker	HE NHLBI
	Dr. Yasuo Nara	Guest Worker	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In a strain of rats bred for their suseptibility to hypertension and stroke (Stroke-prone SHR) we observed a much higher incidence of cerebrovascular lesions when these animals were maintained on a diet containing 15% protein as compared to rats receiving a diet containing 22% protein. The addition of NaCl to the drinking water exacerbated the incidence of stroke particularly in animals receiving a low protein diet. It is suggested that genetic and environmental (dietary) factors interact in a manner that is highly relevant to the incidence of cerebrovascular disease in this animal model.

624

PHS-6040
(Rev. 2-81)

Objectives: Initial observations in our own and in Professor Yamori's laboratory in Japan indicated that in Stroke-prone SHR (SHR-SP) the incidence of stroke was substantially higher in animals receiving commercial Japanese rat food as compared to those receiving the NIH rat formula. This observation formed the basis of a U.S.-Japan cooperative science project. We have subsequently extended these early studies both here and in Japan. The major questions addressed in this study are:

1. Was the previously observed difference in the incidence of cerebral lesions a reproducible phenomenon.

2. Could the Japanese type diet be reproduced in the U.S. and give the same response.

3. Is the difference in protein content of the diets the primary causative agent.

Methods: Groups of SHR-SP were maintained on the NIH-07 rat food (U), or on the Japanese "Funahashi" diet (J), or on a diet prepared in the U.S. according to the Japanese formula (J-U). These rats had blood-pressures and body weights monitored on a routine basis and were observed daily for neurological signs that a cerebral lesion had occurred. Upon death brain were removed and subjected to pathological examination to determine the nature and location of cerebral lesions.

Major Findings: Experiments of this type usually take 6 to 9 months to complete and in the following we will summarize two experiments which have recently been completed. It was reported previously that in a nine month trial in Japan SHR-SP incurred an incidence of stroke or major cerebrovascular lesion of nearly 90% when fed on a commercial Japanese rat diet, whereas similar groups of rats fed the NIH-07 diet had less than a 30% incidence. A repetition of this study was started two years ago and the preliminary results reported last year. In this study, 3 groups of SHR-SP were used. These animals were maintained on the following protocol. Japanese rat diet, U.S. rat diet (NIH-07) and the latter diet plus 1% NaCl. This latter group was included because the (J) diet had a slightly higher Na^+ content than the (U) diet. In this experiment, 28 of 32 rats on the (J) diet had significant cerebral lesions by 9 months of age, whereas only 10 of 34 rats on the (U) diet had such lesions. Rats on the (U) diet + 1% NaCl had an incidence similar to the (U) diet, thus suggesting that Na^+ alone was not responsible for the increased incidence. Both diets, in multiple batches, have been subjected to detailed clinical analysis by commercial analytical laboratories both here and in Japan. No significant differences in vitamin or mineral content were observed with the exception that the Na^+/K^+ ratio was slightly higher in the (J) diet. The most significant difference, however, was in total protein content [(J) = 15% and (U) = 22%]. Hydrolysis of the dietary protein and amino acid analysis revealed no dramatic deficiency or overabundance of any one amino acid. There was a tendency for the sulfur amino acids to be low in the (J) diet. Concurrent studies in Japan revealed that supplementation of the (J) diet with additional protein also had an ameliorating effect on the incidence of cerebral lesions. In order to determine whether perhaps there was some undetected factor in the (J) diet that increased cerebral lesions, we obtained the formula for the (J) diet from the manufacturer and attempted to replicate it from ingredients in the U.S. In a recently completed study, this (J-U) diet was compared with the (J) and (U) diets. The severity of the lesions in the J-U diet appeared to be intermediate between the (J) and (U) diets, but apparently more similar to the (J) diet. In this latter study, the experiments were initiated with animals 6 weeks of age and 1% NaCl was

included in the drinkig water. With this protocol the incidence of stroke was much greater at an earlier age and the time course could be shortened to 3 to 4 months. From these studies we tentatively conclude that the protein content of the rat diet has an important influence on the expression of genetic tendencys for stroke in hypertensive animals.

Significance to Biomedical Research and Institute Programs: The interaction of genetic and environmental factors is increasingly being recognized as a contributor to many diseases. Our current experiments suggest that dietary protein may have an important influence on the expression of stroke in hypertensive aminals. This may have implications for the reduction of mortality and morbidity from cardiovascular disease, which is one of the goals of the institute.

Proposed Course of Project: Experiments are currently underway to determine whether individual amino acid supplements to the (J) or (J-U) diets are effective in reducing the incidence of stroke. We are also evaluating some standard, purified diets as a means of further identifying the components that are effective in preventing stroke.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03529-01 HE

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Phosphorylation Patterns in the Cytosol of the PC12 Pheochromocytoma

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Vivian S. Zabrenetzky	NIH Postdoctoral Fellow	HE NHLBI
OTHER:	Richard MeGee, Jr.	Asst. Prof. Dept. of Pharm. Georgetown Univ.	

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

PC-12 cells contain a cytosolic cAMP-dependent protein kinase that phosphorylates two endogenous substrates as well as histone. Forskolin mimics the effect of cAMP as shown by autoradiography of the substrates. Depolarization of the cells with 150 mM K+ has no effect on basal protein phosphorylation. The addition of Ca2+ causes a decrease from the basal level of phosphorylation in all bands. The addition of calmodulin and Ca2+ restores the level of phosphate incorporation in the bands below 90,000 MW. Nerve growth factor (NGF) induces differentiation of these cells. Cyclic AMP, but not forskolin causes increased phosphorylation.

PHS-6040
(Rev. 2-81)

Objectives: The primary goal was to determine whether there is a phosphorylating system in PC_{12} cells, and whether cAMP or forskolin can effect it.

Methods: PC_{12} cells were grown at Gerogetown University, Department of Pharmacology under the auspices of Dr. Richard McGee Jr. The cells were harvested, centrifuged, incubated under depolarizing (or non-depolarizing) conditions and protein phosphorylation was analyzed after SDS-polyacrylamide gel electrophoresis (PAGE).

Major Findings: The cytosol of PC_{12} cells contains a cyclic AMP-dependent protein kinase that can phosphorylate histone H_1 as exogenous substrate and 2 cytosolic proteins as endogenous substrates. Depolarization of whole cells by 50 mM K^+ had no effect on the phosphorylation pattern. The basal activity of the kinase was not affected by phospholipid. Cyclic AMP causes an increase in phosphate incorporation in two bands. This effect is mimicked by the addition of 5 µM forskolin.. The addition of Ca^{2+} caused a decrease from the basal level of phosphorylation in all bands. The addition of calmodulin and Ca^{2+} restored the level of phosphate incorporation in the bands below 90,000 MW.

The results suggest that there is a cAMP generating system in PC_{12} cytosol and that a cAMP-dependent protein kinase can phosphorylate endogenous substrates.

Significance to Biomedical Research and Institute Programs: PC_{12} cells are from an adrenal pheochromocytoma. This tumor is responsible for a form of hypertension resulting from excessive catecholamines. These cells, unlike other nervous system cells in culture, do not require nerve growth factor (NGF) for survival, rather they undergo differentiation after exposure to NGF. Understanding the biochemistry of these cells is of importance with regard to understanding the role of these cells. Understanding the effect of NGF, as an antimitotic agent, is of importance in understanding the mechanisms of tumor growth.

Proposed Course of Project: The primary goal of this project has been achieved but there are many additional areas to be explored, e.g. the effect of NGF induced differentiation on cofactor and tyrosine hydroxylase activities, and the role of phosphorylation on NGF induction and in possible activation of tyrosine hydroxylase. These are two areas of significance to the research currently underway in this laboratory.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03530-01 HE

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
The Hypotensive Action of 4-(5,6-dimethyl-2-benzofuranyl) piperdine HCl (CGP 6085 A) on Spontaneously Hypertensive and Wilstar-Kyoto rats

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Elliott Kulakowski	Staff Fellow	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sec. Biochem. Pharm.	HE NHLBI
	Jeffery Hurst	Guest Worker	HE NHLBI
	Phyllis Brown	Biol. Lab. Technician	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

CGP 6085 A [4-(5,6-dimethyl-2-bezofuranyl) piperidine HCl] is a serotonin uptake inhibitor, which has been shown to significantly reduce blood pressure in spontaneously hypertensive and normotensive Wistar Kyoto rats. Intraperitoneal administration of CGP 6085 A (10 mg/kg) produces a 30 to 40 percent decrease in blood pressure without altering heart rate. Maximal reduction in blood pressure occurs within one-hour and the hypotensive effect persists for up to three hours. CGP 6085 A produces its dose-dependent hypotensive response over the concentration range of 0.1 to 10 mg/kg. This depressor response corresponds to the ability of CGP 6085 A to inhibit uptake of 4-methyl-β-ethylphenylamine (H75/12). H75/12, which is taken up by the serotonin uptake system and depletes intraneuronal 5HT stores was used to measure the ability of CGP 6085 A to block depletion of 5HT by H75/12. CGP 6085 A blocks the 5HT depletor action of H75/12 in a dose-dependent manner. No changes in pons-medulla norepinephrine or dopamine levels were observed.

629

PHS-6040
(Rev. 2-81)

Objectives: CGP 6085 A has previously been shown to block the uptake of 5HT into brainstem neurons. We decided to investigate the possibility that CGP 6085 A could produce a depressor response in SH and WKY rats. These studies consisted of 1) a time course of the effects of CGP 6085 A in reducing blood pressure, 2) a dose response curve of the hypotensive effects of CGP 6085 A, 3) an attempt to correlate the depressor actions of CGP 6085 A with its ability to block uptake of various biogenic amines.

Methods: Male rats of the Wistar Kyoto (WKY) and spontaeously hypertensive (SHR) strains were bred at the Small Animal Section, Veterinary Resources Branch, Division of Research Services, National Institutes of Health, Bethesda, MD. The animals were maintained in laminar flow units on a twelve hour light-dark cycle at 24°C and constant humidity. Food and water were available ad libitum.

Arterial blood pressure was determined from the caudal artery in unanesthetized animals by a modification of the tail cuff method. Heart rate was determined simultaneously from the pulse tracings. The animals were prewarmed at 37°C for 7-10 minutes prior to each reading. For each time point, at least three measurements were taken. Resting blood pressure in the normotensive WKY rats was less than 140 mm Hg and was greater than 150 mm Hg in the SHR rats. Aftter control blood pressures were measured, the animals were injected with the indicated amount of CGP 6085 A. Blood pressure and pulse rates were recorded at the times indicated.

Determination of the ability of CGP 6085 A to inhibit serotonin uptake in vivo was performed indirectly by use of H 75/12, a serotonin depletor. Experiments in which serotonin levels were measured one hour following CGP 6085 A administrations systolic blood pressure was recorded and H 75/12 (50 mg/kg) was injected intraperitoneally. Fifty minutes later the animals were sacrificed and brainstems were removed and frozen for assay of indole alkyl amines. H 75/12 previously has been shown to be taken up by the serotonin transport system and to specifically deplete neuronal serotonin. However, pretreatment of animals with CGP 6085 A was shown to inhibit the uptake of H 75/12. Thus, the amount of 5HT remaining in the brainstem is a measure of the ability of CGP 6085 to block H 75/12 uptake.

Brains were rapidy dissected on ice and brainstems were weighed, frozen and stored at -80°C for analysis of 5HT and 5HIAA by high-performance liquid chromatography with electrochemical detection (HPLC-EC). The brainstems were weighed and homogenized in 1.0 ml 0.1 N percholoric acid containing 0.02% ascorbic acid and 6-fluoroserotonin (6-F-5-HT), to serve as the internal sandard, and centrifuged for 10 min at 1000 x g to obtain a protein-free supernatant. The amounts of 5-HT and 5-HIAA were determined by high pressure liquid chromatography. An aliquot of the protein-free supernatant was recentrifuged briefly and 50 ul were injected into the HPLC apparatus consisting of an Altex 110 A pump, Altex 210 injector, uBondapak C_{18} column (3.9 x 200 mm, Waters Associates), an LC4A electrochemical detector with a glassy carbon electrode (Bioanalytical Systems Inc.) and a linear strip chart recorder. The mobile phase consisted of 0.08 M sodium acetate (titrated to pH 4.89 with glacial acetic acid) with 2.5 mM Na_2 EDTA, 2% (v/v) methanol and 4% acetonitrile. With a flow rate of 1.4 ml/min, separation of sample components was achieved within 11 minutes. Detector potential was set at 0.50 V vs Ag/AgCl, which eliminated all interfering peaks near the elution time of the substances of interest. Quantitation was based on peak height measurement and ratios of 5-HT and 5-HIAA to the internal standard, 6-F-5-HT.

Concentrations of NE, DA and DOPAC were determined by extracting 500 ul of the protein-free supernatant with 10 mg of alumina, and eluting catechols into 100 ul of 0.2 N PCA. 25 ul of the eluate were injected into the HPLC aparatus, consisting of an M 6000 pump, U6K injector, a Radial-Pad C_{18} cartridge (8 x 100 mm 5 um particle size) in a Radial compression Module (all from Waters Associates) an electrochemical detector similar to the one described above and a dual-channel chart recorder (Sargent-Welch). The mobile phase consisted of 0.1 M triethylamine, 0.1 N phosphoric acid, 2.5 mM Na_2 EDTA, 6 mM heptanesulfonic acid and 7% (v/v) aectonitrile, and was pumped at a rate of 1.4 ml/min. This resulted in a separation of the substances of interest in 12 min. Detector potential was set at 0.60 V vs Ag/AgCl. Quantitation was based on peak hight measurement and ratios of NE, DA, and DOPAC to the internal standard, NMDA.

Major Findings: Intraperitoneal injection of CGP 6085 A (10 mg/kg) into SHR produced a significant decrease in systolic blood pressure when compared to vehicle injections. The onset of action occurred within thirty minutes and a maximum reduction in blood pressure of 30-40% was maintained for up to three hours. The duration of action of CGP 6085 A is less than five hours. While CGP 6085 A produced a dramatic decrease in blood pressure, no alteration in resting pulse rate was observed.

CGP 6085 A produced a similar reduction in systolic blood pressure in the WKY rats. We observed a decrease in blood pressure of approximately 40-50 mm Hg and control levels were achieved five hours after drug administration. However, the onset of action appears to be slightly retarded, though maximal blood pressure decrease is observed within one hour.

The depressor response of CGP 6085 A in SH and WKY rats is dose dependent. Over the concentration range of 0.1-10 mg/kg rat, all doses of CGP 6085 A produced decreases in systolic blood pressure when measured one hour following drug administration. The maximal effect of CGP 6085 A in both normotensive and hypertensive animals is attained at a dose of 3 mg/kg.

H 75/12, a specific serotonin depletor, was utilized as a tool to measure the dose response effects of CGP 6085 A in blocking 5HT uptake. Intraperitoneal administration of H 75/12 was examined over the range of 1-50 mg/kg. Fifty minutes after administration of H 75/12, characteristic locomotor and behavioral signs of serotonin depletion were observed. At doses greater than 10 mg/kg H 75/12 produce piloerection, straub tail, backward walking and front paw treading. Corresponding to these changes there is a dose-dependent decrease in brainstem 5 5HT levels. No alterations in 5HIAA levels are seen.

It is believed that H 75/12 is taken up into neurons by a serotonin transport system. Since CGP 6085 A inhibits the 5HT uptake system, treatment with this drug should prevent neuronal 5HT release by blocking H 75/12 uptake into the neuron. Prior administration of CGP 6085 A blocks H 75/12 induced depletion of 5 HT from the caudal brainstem of both SHR and WKY animals in a dose-dependent manner. In both SHR and WKY 10 mg/kg CGP 6085 A was able to effectively block H 75/12 depletion of brainstem 5HT. Similarly, CGP 6085 A was able to attenuate the locomotor and behavior changes induced by H 75/12 in a dose dependent manner. At doses greater than 3 mg/kg, these effects were abolished. The decrease in 5HIAA levels observed at higher doses of CGP 6085 A is apparently due to the effects of this compound since no reduction in 5HIAA was observed in the presence of H 75/12 alone.

Since central serotonergic neurons have been associated with a decrease in blood pressure, we sought to determine if the depressor response of CGP 6085 A is related to the drugs ability to block 5HT transport. The correlation coefficient for the WKY and SHR animals was 0.75 and 0.71, respectively. Though blood pressures were measured fifty minutes before the administration of H 75/12, the data appears valid since no significant changes in blood pressure were observed over this time course.

Significance to Biomedical Research and Institute Programs:

1. CGP 6085 A has a depressor response in blood pressure in SHR and WKY rats.

2. The hypotensive action of CGP 6085 A is dose dependent.

3. This dose dependent reduction in blood pressure by CGP 6085 A correlates with its ability to inhibit serotonin uptake without affecting transport of norepinephrine, dopamine and DOPAC.

Proposed Course of Project: Initial observations into the cardiovascular acion of CGP 6085 A have been initiated. We propose to examine the central effects of CGP 6085 A by injecting CGP 6085 A into the lateral ventricles and raphe nucleus and determine its effect on blood pressure. We also plan to examine the peripheral effects of CGP 6085 A in altering vascular tone. Finally, we plan to examine the chronic effects of CGP 6085 A to determine if this drug lowers the basal blood pressure levels or produces tachyphylaxis in the animal models studied.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03531-01 HE

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Tryptophan Hydroxylase: Purification by Affinity Chromatography on Calmodulin-Sepharose

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Donald M. Kuhn	Staff Fellow	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharml.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Tryptophan hydroxylase from rat mesencephalic tegmentum has been purified by sequential chromatography on Blue-Sepharose, DEAE, and calmodulin-Sepharose. The hydroxylase is excluded from Blue-Sepharose and is eluted from DEAE with a stepwise NaCl gradient. Finally, tryptophan hydroxylase binds to calmodulin-sepharose and is eluted with EGTA. The purification scheme is rapid and yields an enzyme with a specific activity of 3.5 nmol 5HTP/mg min, representing a 61-fold purification with 8% recovery.

PHS-6040
(Rev. 2-81)

Objectives: Tryptophan hydroxylase [EC 1.14.16.4; ℓ-tryptophan, tetrahydropterin: oxygen oxidoreductase (5-hydroxylating)] catalyzes the initial and rate-limiting step in the biosynthesis of the neurotransmitter serotonin. Highly purified forms of tryptophan hydroxylase have been difficult to obtain because of the extreme lability of this enzyme, a property of this hydroxylase which has been attributed to its thermo-oxidative sensitivity (Kuhn et al., JBC, 4137-4143, 1980).

We recently reported that tryptophan hydroxylase is activated in vitro by a calcium-calmodulin (CaM)-dependent protein kinase (Kuhn et al., PNAS, 4688-4691, 1980) and, during the course of experiments designed to purify this protein kinase, we observed that tryptophan hydroxylase is also a CaM-binding protein. As a result of this unique property of tryptophan hydroxylase, an attempt to purify the enzyme by affinity chromatography on CaM-Sepharose has been made.

Methods: Tryptophan hydroxylase was assayed in rat mesencephalic tegmental extracts and column fractions by the method of Baumgarten et al. (J. Neurochem. 21:251, 1973).

Blue-Sepharose chromatography was carried out as described by Morrill et al. (JBC, 254:4371, 1979). CaM was purified from bovine brain by the method of Charbonneau and Cormier (BBRC, 90:1039-1047, 1979) with slight modifications. In short, fluphenazine was coupled to diglycidyl ether-activated Sepharose by dissolving the powder in distilled water and the drug solution was added drop wise to activated Sepharose with gentle heating. CaM-Sepharose was prepared by the method of Klee and Krinks (Biochem. 17:120-126m 1978) and contained 3 mg of CaM/ml of Sepharose 4B.

Major Findings: Supernatant fractions from tegmental extracts were applied to a 1.0 x 5.0 cm Blue-Sepharose column equilibrated with 50 mM Hepes pH 7.4 containing 2 mM DTT, 0.05 M NaCl, 200 uM Ca^{2+}, 1 mM Mg^{2+} and 15% (v/v) glycerol. Tryptophan hydroxylase is excluded from Blue-Sepharose but this chromatographic step was chosen since many proteins which bind to CaM-Sepharose (see below) also bind to Blue-Sepharose (e.g., CaM-dependent phosphodiesterase). Furthermore, Mg^{2+} was included in the equilibration buffer since this divalent ion enhances the binding of at least CaM-dependent phosphodiesterase and CaM-dependent protein kinase to Blue-Sepharose.

Fractions from the Blue-Sepharose column containing tryptophan hydroxylase were pooled and applied to a 1.0 x 6.0 cm column of DEAE equilibrated with 50 mM Hepes pH 7.4 containing 2 mM DTT, 0.05 M NaCl, 200 uM Ca^{2+}, 50 uM EDTA, and 15% glycerol. The DEAE column was washed with equilibration buffer until fractions were devoid of protein. Tryptophan hydroxylase was then eluted with 0.15 M NaCl in equilibrating buffer. Fractions from the DEAE column containing enzyme activity were pooled and catalase was added to achieve a concentration of 0.04 mg/ml. The pooled fraction was then applied to a 1.5 x 8 cm column of CaM-Sepharose equilibrated with 50 mM Hepes pH 7.4, 2 mM DTT, 200 uM Ca^{2+}, 0.15 M NaCl, 50 uM EDTA, 15% glycerol and 0.04 mg/ml catalase and the CaM-Sepharose column was washed with equilibrating buffer until fractions were devoid of protein other than catalase. Tryptophan hydroxylase was then eluted from the CaM-Sepharose column by replacing the Ca^{2+} in the equilibrating buffer with 1.0 mM EGTA. Tryptophan hydroxylase can also be eluted from CaM-Sepharose with CaM (50 uM) but apparently not with ℓ-tryptophan (up to 50 mM).

The fold purification from this purification scheme is relatively small (61-

fold) and probably represents an underestimation since the eluted enzyme loses catalytic activity rapidly (e.g., after only 24 hr storage at 4°C, activity is reduced over 80%). Catalase was the only substituent which appeared to stabilize tryptophan hydroxylase during purification and in the absence of catalase almost no activity was eluted from CaM-Sepharose. Since the entire purification scheme from homogenization of brain tissue to assay takes only 5-6 hr, stabilization of tryptophan hydroxylase during purification remains a significant problem.

Although tryptophan hydroxylase binds to CaM-Sepharose, CaM itself has no apparent effect on tryptophan hydroxylase activity. The addition of CaM (up to 20 uM) to tegmental extracts which have been chromatographed on fluphenazine-Sepharose to remove endogenous CaM does not alter hydroxylase activity nor does CaM alter the stability of tryptophan hydroxylase.

Significance to Biomedical Research and Institute Program: This purification scheme is the first one devised to yield a highly purified preparation of brain tryptophan hydroxylase. Application of this method will now allow a number of important experiments to be carried out concerning the control of tryptophan hydroxylase activity, especially the effect of phosphorylation on enzyme activity. Furthermore, antibodies to tryptophan hydroxylase will be important in contributing to a clearer understanding of the cellular localization of tryptophan hydroxylase (and hence, serotonin).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03532-01 HE

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Glucocorticoid regulation of tetrahydrobiopterin in PC-12 cells.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Anne Culvenor	Guest Worker Fogarty International Center	HE NHLBI
OTHER:	Vivian Zabrenetzky	Postdoctoral Fellow	HE NHLBI
	Richard MeGee	Asst. Prof., Dept. Pharmacology	Georgetown Univ.
	Walter Lovenberg	Chief, Sec. Biochem. Pharm.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5		

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

PC-12 cells have been established in the laboratory as a continuous culture. The cells contain large amounts of biopterin, with > 95% being present as tetrahydrobiopterin (BH4).

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PHS-6040
(Rev. 2-81)

Objectives: The main aim of this project is to investigate whether BH_4 levels in PC-12 cells are regulated in parallel with tyrosine hydroxylase (TH) levels. Glucocorticoids such as dexamethasone are known to induce TH in PC-12 cells. The effects of glucocorticoids on BH_4 and TH in PC-12 cells will therefore be studied.

Methods: PC-12 cells obtained from Dr. R. McGee and Dr. G. Guroff were maintained in Dulbecco's modified Eagle medium containing 10% fetal calf serum and 5% horse serum in an atmosphere of 10% CO_2 in air at 36°C. Reduced and oxidized biopterin in cell homogenates was assayed by differential iodine oxidation and high performance liquid chromatography (Fukushima and Nixon, Anal. Biochem. 102, 176, 1980).

Major Findings: High levels of BH_4 are present in PC-12 cell homogenates (about 30 ng BH_4/mg protein). Most of the total biopterin is present as BH_4. Biopterin was present in the growth medium of the cells, presumably released as one of the reduced or oxidized forms of biopterin.

Significance to Biomedical Research and Institute Programs: PC-12 cells should provide a useful model system for investigating the regulation of BH_4 levels, especially in relation to TH levels and monoamine synthesis.

Proposed Course of Project: The principal investigator terminates her fellowship at the end of August 1982. In the three months remaining, assay conditions for measurement of TH activity in PC-12 cells will be established. Cell cultures will then be exposed to glucocorticoids and the effects on levels of BH_4 and TH studied.

ANNUAL REPORT OF THE
LABORATORY OF KIDNEY AND ELECTROLYTE METABOLISM
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1981 through September 30, 1982

Our continuing goal is to analyze the function of the kidney as a basis for understanding its pathophysiology and treating its disorders. Since the formation of urine depends upon the transport of water and solutes by kidney tubules, understanding renal function requires analysis of these cellular processes and of their integration in the kidney. Therefore, we are studying transport by cells in general and kidney cells in particular, as well as the mechanisms, hormonal and other, that control transport.

Isolated segments of renal tubules.

In order to understand kidneys on the cellular and molecular level the functions of the different types of epithelial cells must be identified. Progress in this direction has relied heavily on the direct study of individual nephron segments. Each nephron segment has a different cell morphology and function. An important method (which originated in this laboratory) for directly studying the nephron segments is to dissect them and perfuse them individually in vitro. The findings, during the past year, using this method are as follows:

Urea is thought to play a central role in the renal concentrating mechanism by accumulating in high concentration in the renal medulla. Consequently, it is important to understand the pathways of urea addition to the medulla. To accomplish this, Knepper is measuring urea permeability and transport directly in nephron segments dissected from rat kidney. Contrary to prior theory, he finds that rat cortical thick ascending limbs are permeable to urea (and do not transport it actively). He also finds that proximal straight tubules (which lie adjacent to thick ascending limbs in the cortical medullary rays) are are also permeable to urea. He proposes that urea carried from the medulla in high concentration in thick ascending limbs diffuses from them into adjacent proximal straight tubules which in turn carry the urea back into the medulla. His theoretical analysis demonstrates that this newly discovered cycle should contribute to urinary concentration and urea excretion. He plans further studies to complete the survey of urea transport along the rat nephron and to test a number of theoretical models which have been recently proposed.

Kallikrein is believed to be important in renal sodium excretion and hypertension, but its exact role in the kidney is not known. In order to determine directly where the enzyme acts in the kidney, Knepper, Proud, Pisano, and Burg have measured by radioimmunoassay the kallikrein content of individual nephron segments dissected from rats. The highest content was found in connecting tubules. Significant quantities were also found in distal convoluted tubules, initial collecting tubules, and cortical collecting ducts. The next step should be to perfuse the tubules in vitro in order to test the role of the kallikrein system, but it had not previously been practical to dissect rat tubules without collagenase, and collagenase weakens the tubule basement membranes enough to prevent perfusion. Knepper, however, has recently discovered that cortical collecting ducts can be dissected from pathogen-free rats without collagenase and perfused successfully. He plans to study the role of kinins and various hormones including vasopressin and aldosterone in the regulation of electrolyte transport by collecting ducts and other segments.

The pH of the urine is an important determinant of how much acid is excreted by the kidney. Final adjustment of the urine pH occurs in the collecting ducts. We previously measured bicarbonate reabsorption and pH in proximal tubules and cortical collecting ducts from rabbits. Atkins and Burg are now measuring acid-base transport by collecting ducts from rats, using Knepper's new preparation. Initially, the investigators measured the pH of fluid collected from each of the the three parts of the collecting duct (cortical, outer medullary, and inner medullary) perfused in vitro. At slow perfusion rates pH of collected fluid was lower than in the perfusate and bath. Surprisingly, however, the the fall in pH was considerably greater when the bathing fluid was free of bicarbonate. The pH of fluid collected from inner medullary collecting ducts (which are the terminal nephron segments) was not generally as low as that in the bladder urine of the rats from which the tubules were dissected. A number of variables were tested to see if additional acidification could be stimulated, but none led to a lower pH. At present the factors affecting the generation of pH gradients in the collecting ducts are being studied further.

Ammonia synthesized within kidney cells provides the major urinary buffer for excretion of acid, and its rate of synthesis increases greatly during metabolic acidosis. It is not known, however, exactly which nephron segments produce the ammonia and how it goes from them to the final urine. Therefore, Good, Burg, and Vurek have been measuring in vitro ammonia production by individual nephron segments dissected from rat kidney. In order to do this they developed a sensitive microfluorometric assay for picomole quantities of ammonia. They incubated kidney tubule segments in vitro and demonstrated that proximal tubules, thick ascending limbs and collecting ducts from rats in normal acid-base balance all synthesized ammonia. In rats made chronically acidotic by pretreatment with ammonium chloride, however, ammonia production increased only in proximal tubules. The investigators are completing their survey of nephron segments, then plan to adapt the method to measure ammonia permeability and transport in isolated perfused tubules in order to find out how the ammonia that is produced reaches the urine.

Cell culture of epithelia.

Although the technique of perfusing kidney tubules in vitro has provided an overall description of their transport properties, it has been difficult to extend the studies to subcellular and molecular levels. Chemical and physical methods for studying transport require much larger amounts of homogeneous tissue than are present in single tubules. Dr. Handler and his colleagues have been using cultures of epithelial cells to overcome this difficulty.

Moran and Turner have completed their initial characterization of the apical membrane sodium coupled glucose transporter in LLC-PK1 which is a line of epithelial cells cultured from pig kidney. The rate of transport in apical membrane vesicles parallels the transport in intact cultured epithelia (assessed as the steady state concentration of the transported sugar in the epithelium). Of interest is that the rate of sugar transport is regulated by the concentration of glucose in the medium in which the cells have been grown. Epithelial cells grown in low concentrations of glucose develop high rates of transport, epithelia grown in high concentrations of glucose develop low rates of transport. The effect of a change in the glucose concentration is not evident for at least 24 hours, and can be reversed by reversing the glucose concentration in the medium. The mechanism of this regulatory effect is being studied further.

Handler et. al. have continued studying epithelia formed by A6 cells, a continuous line derived from the kidney of Xenopus laevis. Previously they found that epithelium functions similarly to the collecting duct. Now they are studying two aspects of hormone action on the epithelia. Previously they found that the epithelium increases its sodium transport in response to adrenergic agents, adenosine, vasopressin, insulin and aldosterone. This occurs only if it is grown on filter bottom cups that allow access of medium to the basal surface of the cells, but not if the cells are grown on solid dishes (see last years report). Preston and Handler now find by using standard agonists and antagonists that the response to adrenergic agents is beta adrenergic. Also, Lang, Forrest, Preston and Handler now find that the response to adenosine is a ribose agonist effect. Since epithelia grown on petri dishes did not previously respond measurably to vasopressin, isoproterenol, or adenosine as do those in filter bottom cups, Lang, Forrest, and Preston are now studying the nature of the development of responsiveness on filter bottom cups. They will assess the development of specific binding to hormone receptors, as well as receptor mediated stimulation of adenylate cyclase and sodium transport.

Sohraby, Burg, and Turner are characterizing the sodium channel in the membrane of A6 cells. It was previously shown that the epithelium formed by these cells maintains a transepithelial voltage oriented apical surface negative, a high transepithelial resistance, and active transepithelial sodium transport. The sodium transport by these cells resembles that of renal collecting ducts and toad bladders. In the latter tissues transported sodium is believed to enter the cells by a special channel through their apical membranes. Characterization of this channel could be facilitated by its study in cell culture. Therefore, the investigators have now measured sodium flux across the apical membranes of the A6 cells by determining the initial rate of uptake of ^{22}Na from the apical bath. This sodium flux was stimulated three-fold by incubation with aldosterone. Amiloride inhibited both the basal and the aldosterone stimulated flux in a competitive fashion. In both respects the A6 cells closely resemble renal collecting ducts. With this information as a background the investigators next plan to prepare vesicles from the the apical membranes of these cells.

Transport of D-glucose.

D-glucose is reabsorbed from the urine by the renal proximal tubule. The active step is D-glucose co-transport with sodium into the cells across the lumen brush border membrane. Turner et al are studying this process in membrane vesicles since, in addition to its obvious physiological significance, the brush border membrane D-glucose co-transporter is a useful model system for studying the mechanism of cotransport processes in general.

Recently several primary active transport proteins have been extracted from cell membranes and purified to near homogeneity by conventional protein separation techniques. Coupled transporters, however, have for the most part eluded investigators who have attempted this approach. One reason for this is that, unlike primary active systems, coupled transporters do not typically exhibit an easily measured enzymatic activity which would allow one to follow them through the various extraction and separation steps. Turner and his coworkers are attempting to circumvent this problem by making use of a special property of the sodium-coupled D-glucose transporter. That is, Turner and George, have now found an essential disulfide bond on this transporter which

when reduced by dithiotreitol results in a irreversible inactivation of phlorizin binding and glucose transport. Since substrates of the carrier protect against inactivation by dithiotreitol, this disulfide bond is apparently close to the glucose binding site.

Walter and Turner are attempting to label the transporter by making use of above the observations. They are incubating membranes with dithiotreitol in the presence or absence of protecting sugars, then tagging the sulfhydryl groups resulting from reduction of disulfide bonds with radiolabeled N-ethylmaleimide. The degree of labelling of the transporter is different in protected and unprotected preparations. They find in their initial studies that they can identify the relevant protein bands using polyacrilamide gel electrophoresis. Having identified these proteins, they plan to isolate sufficient material (using preparative electrophoresis) for reconstitution and immunological (monoclonal antibodies) studies.

All of the estimates of the molecular weight of the D-glucose transporter in the literature have been indirect, and considerable disagreement exists. Turner and Kempner have now determined the molecular weight of the transporter by monitoring the phlorizin binding of brush border membrane vesicles as a function of radiation dose. The rate at which an enzyme is inactivated by radiation is proportional to its volume (target size) and hence its mass. They find a molecular weight of approximately 110,000 daltons.

Turner and Moran are studying glucose transport in brush border membrane vesicles prepared from the outer cortex and outer medulla of rabbit kidney. They observe a low affinity, high capacity transporter in the outer cortical preparation and a high affinity, low capacity transporter in the outer medulla. Since the outer cortical and outer medullary preparations contain predominantly BBM from early and late proximal tubules respectively, there must be D-glucose transport heterogeneity along the length of the proximal tubule. The outer medullary transporter is almost two orders of magnitude less sensitive to phlorizin than the outer cortical one. They devised a new method for measuring the activator:substrate stoichiometry for coupled transport systems in vesicle preparations. The stoichiometry of the outer cortical transporter is 1:1 (sodium:glucose). The stoichiometry of the outer medullary system is approximately 2:1. This arrangement of transporters enables the kidney to reabsorb glucose from the urine in an energy efficient fashion. The bulk of the glucose is reabsorbed early in the proximal tubule at an energetic cost of one sodium ion per glucose molecule. Then in the late proximal tubule a larger coupling ratio (2:1) and hence a larger driving force reabsorbs the last traces of glucose from the urine.

Turner has carried out several theoretical analyses of cotransport models. He demonstrated that the general flux equation for the rapid equilibrium carrier model of cotransport can be written entirely in terms of five independent kinetic constants. This result was used in turn to derive a series of rejection criteria for the model. In order to extend his theoretical studies to steady-state models a computer program has been written which is capable of solving an arbitrarily complex kinetic model.

Necturus gallbladder epithelium.

The cellular aspects of epithelial transport can be studied by making measurements in individual cells. Since mammalian cells generally are very small, such direct studies are difficult and there are artefacts due to cell damage. Therefore, Spring and coworkers have been using the very large gallbladder cells of Necturus for this purpose.

Larson and Spring have been studying the uptake of NaCl into gallbladder epithelial cells across the apical cell membranes by measuring cell volume changes that occur under special conditions. Thus, they used ouabain to specifically inhibit NaCl transport out of the cells across their basal membranes. Then, the cells swelled progressively because of continued entry of salt and water across their apical border. The rate of swelling equalled the normal rate of transepithelial salt and water transport. By measuring the effect of variables on the rate of swelling they were able to characterize the apical transport uptake step. A quantative light microscopic method described in previous reports was used for the measurements of volume. They found that the diuretic drug bumetanide inhibited this entry step rapidily and reversibly. When bumetanide was applied in the absence of ouabain, the cells shrank and the activities of sodium and chloride in the cells decreased. Having identified bumetanide as a potent inhibitor of the sodium chloride entry step at the lumen membrane, the investigators intend to use this drug to further characterize the system.

Spring and his coworkers previously observed that when Necturus gall bladder cells shrank or swelled in anisotonic media, the cells soon returned to their initial volume, despite continuation of the anisotonic conditions. The investigators are now characterizing these responses, using measurements of cell volume and ion specific electrode measurements of cell ion activity. The regulatory increase in volume in shrunken cells was described in last year's report. In brief it involves NaCl uptake across the apical membranes of the cells by parallel Na^+/H^+ and Cl^-/HCO_3^- exchange. It is important to note that this mechanism is different from the directly coupled NaCl uptake that is involved in normal transepithelial salt transport. Bumetanide did not inhibit the volume regulatory increase as it did the normal coupled NaCl transport. The investigators have now found that the regulatory decrease in cell volume involves yet another ion transport mechanism. Decrease in volume of swollen cells results from coupled loss of potassium and chloride followed by osmotic flux of water. The loss of KCl may well be across only the basal cell membranes, judging from the initial studies. Bumetanide inhibits this process, and it appears that the drug may be a general inhibitor of directly coupled transport processes.

Metabolism associated with solute transport.

Balaban and his coworkers have been studying the sources of metabolic energy for transport and coupling between metabolism and transport in intact cells. In order to accomplish this they have been developing and utilizing new non-invasive techniques for monitoring metabolism and transport in intact tissues and cells.

Balaban and Bader have estimated the efficiency of Na-K-ATPase (i.e. the number of potassium ions transported per ATP hydrolized) in intact transformed

cells. They are using extracellular ion and gas sensitive electrodes for their measurements. They find that the Na-K-ATPase is fully as efficient in transformed as in normal cells, contrary to recently published views. In addition, they find that the Na-K-ATPase turnover is more closely coupled to the rate of aerobic glycolysis than to oxidative metabolism. The latter result suggests a specially close coupling between ion transport and glycolysis. The basis of this coupling is being investigated.

Glycolysis is an important source of energy for transport in kidney medulla, brain, and skeletal muscle, as well as in cultured cells. Therefore, Balaban and Walter are characterizing the efflux of the end products of glycolysis, i.e. lactate and protons, out of metabolizing cells across their plasma membranes. They are measuring intracellular pH and lactate concentration, membrane potential, and lactate and proton efflux rates in intact cultured cells by a combination of techniques, including optical spectroscopy, NMR, and ion and gas sensitive electrodes. At this time the methods have been successfully developed and the initial measurements are being made.

Nuclear magnetic resonance (NMR) is another promising non-invasive method for measuring intracellular concentrations and and chemical reaction rates of metabolic intermediates. It also can measure transport fluxes across cell membranes by the use of shift reagents. With use of ^{31}P NMR one can measure ATP, inorganic phosphate, creatine phosphate, ADP, and sugar phosphates in tissues in vivo. Balaban is using ^{31}P NMR to investigate the effects of hypoxia, hypovolemic shock and exercise on the levels of these compounds in rat brain and kidney. In addition Balaban and Knepper have demonstrated that ^{14}N NMR distinguishes free ammonia, choline compounds and urea in vivo. In order to extend the biological application of NMR, Balaban, Knazek, and Sohraby are modifying the dialysis fiber system developed by Knazek to grow a sufficient density of epithelial cells in culture for study by NMR. Also, Balaban and Ferretti are developing two dimensional (2D) NMR techniques to determine rate constants of metabolic reactions under steady state conditions in intact cells. In order to verify the 2D NMR method they are initially measuring enzymatic rate constants in vitro for phosphoglucose isomerase, adenylate kinase and creatine kinase. These in vitro experiments have not only demonstrated the validity of the technique, but have provided new information about the kinetic behavior of the enzymes at equilibrium. The 2D NMR technique will next be applied to intact cell preparations in which the investigators will attempt to measure the unidirectional rate constants of biochemical reactions involving high energy phosphate compounds.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01217-07 KE

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Epithelial fluid transport and morphology

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Kenneth R. Spring, DMD, Ph.D.	Physiologist	LKEM	NHLBI
Other:	Mikael Larson, Ph.D.	Visiting Fellow	LKEM	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	0.25	1.00

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Epithelial cells swell when the Na-K pump is inhibited with ouabain. The swelling is due to NaCl entry into the cell across the apical membrane. NaCl entry into the cell was shown to be tightly coupled and carrier mediated. It would be inhibited by the diuretic, Bumetanide. The kinetics of this inhibition were characterized.

644

PHS-6040
(Rev. 2-81)

Objectives

The primary goal of this investigation is elucidation of the mechanism of fluid absorption of epithelia. The methods employed represent a unique blend of optical and electrical techniques developed specifically for this investigation. We use an online, real time system for measuring the size and shape of the cells. Based on changes in cell volume we are able to determine the mechanism and rate of entry of NaCl into epithelial cells across the apical membrane.

Methods

The gallbladder of the amphibian Necturus maculosus is mounted in a chamber designed to allow the continuous perfusion of both surfaces of the tissue, measurement of epithelial electrical properties, variation in transepithelial hydrostatic pressure, and rapid alteration of perfusate composition. The chamber is placed on the stage of an inverted microscope. Position and focus of the microscope are monitored by observation of the preparation during measurements with the aid of a television camera. The video images are stored on a disc recorder and later analyzed by tracing cell outlines. Cell size and shape are determined from video records obtained during alteration in perfusate composition or other experimental manipulations.

Major Findings

The rate of NaCl entry into gallbladder epithelial cells was determined by measurement of the rate of cell swelling following inhibition of the cell Na-K ATPase by application of ouabain. The swelling was caused by NaCl entry into the cells across the apical membrane because it could be prevented by lowering the Na or Cl concentration of the bathing solution to 10 mM. The diuretic, Bumetanide, inhibited NaCl entry into the cells and prevented cell swelling. Bumetanide was shown to be a reversible, noncompetitive inhibitor of NaCl cotransport; half maximal inhibition was achieved at 1×10^{-9} M.

Proposed Course

Since coupled NaCl transport in other systems involves K, we will test for a K requirement. We will measure intracellular ion activities during inhibition of transport by Bumetanide to determine the flow of salts into and out of the cell.

Publications

M. Larson and K.R. Spring: Bumetanide inhibition of neutral NaCl transport by Necturus gallbladder epithelium. Fed. Proc. 41(4):1495, 1982.

A.-C. Ericson and K.R. Spring: Coupled NaCl entry into Necturus gallbladder epithelial cells. Am. J. Physiol,:Cell (in press), 1982.

A.-C. Ericson and K.R. Spring: Volume regulation by Necturus gallbladder: Hypertonicity activates apical Na-H and Cl-HCO_3 exchange. Am. J. Physiol.: Cell (in press), 1982.

K.R. Spring and A.-C. Ericson: Epithelial cell volume modulation and regulation. J. Memb. Biol. (in press), 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01224-06 KE

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Control of sodium and potassium transport in the nephron

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Mark Knepper, M.D., Ph.D.	Staff Associate	LKEM	NHLBI
Others:	Maurice B. Burg, M.D.	Chief, LKEM	LKEM	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, Bethesda, MD 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Experiments were conducted to localize kallikrein along the rat nephron using radioimmunoassay. The highest activity per unit length was in the connecting tubule. A significant content was also found in distal convoluted tubules, initial collecting tubules, and cortical collecting ducts. These data provide information which will aid the rational design of in vitro perfusion experiments to test the role of the kallikrein-kinin system in the regulation of NaCl and water transport in the kidney.

647

PHS-6040
(Rev. 2-81)

Objectives

The major goal of this investigation is to define the role of the kallikrein-kinin system in the regulation of NaCl and water transport in the kidney. A preliminary objective is to identify which segments of the nephron have intracellular kallikrein and to determine the role of mineralocorticoids, if any, in the regulation of renal kallikrein content.

Methods

A radioimmunoassay technique was used for the kallikrein localization studies. Preliminary localization studies were done on lyophilized kidney slices from various levels along the cortico-papillary axis from the kidneys of control rats and rats treated either with deoxycorticosterone or dietary sodium restriction. Further studies were done in microdissected nephron segments from rats. The kidneys were traeated with collagenase to allow dissection of long lengths of each nephron segment from the renal cortex.

Major Findings

In the slice studies, the kallikrein content was found to be highest in the slices from the outer cortex. There was a progressive decrease in kallikrein content per unit dry weight from the outer cortex to the papillary tip. The kallikrein content was significantly increased in cortical slices from rats given a low sodium chloride diet.

In microdissected tubules, the kallikrein content was found to be highest in connecting tubules (155 + 31 [s.e.] pg/mm tubule length). Significant quantities of kallikrein were also found in initial collecting tubules, distal convoluted tubules, and cortical collecting ducts. Little or no kallikrein was found in glomeruli, proximal convoluted tubules, proximal straight tubules, and cortical thick ascending limbs. There was no demonstrable change in kallikrein content of any tubule segment in response to dietary salt restriction or deoxycorticosterone administration.

Proposed Course

Studies in isolated cortical collecting ducts from rats are planned to test the effects of a number of hormones on sodium and water transport. The ultimate goals are to define the role of this nephron segment in overall regulation of NaCl excretion by the kidney and to define the mechanisms of action of the hormones. Major emphasis will be on aldosterone and vasopressin. In addition, kinins and catecholamines may have important effects in this segment.

Significance

The understanding of the control of water and electrolyte excretion by the kidney depends on knowledge of control mechanisms in the component parts of the kidney, i.e. the nephrons. An integrated view of control of electrolyte excretion by the kidney is crucial to the understanding of the pathophysiology of hypertension and other pathologic states.

Objectives

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Proposed Course

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Significance

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Publications

M.A. Knepper and M.B. Burg. Increased fluid absorption and cell volume in isolated rabbit proximal straight tubules following in vivo DOCA administration. Am. J. Physiol. 241:F502-F508. 1982.

D. Proud, M.A. Knepper, and J.J. Pisano. Distribution of immunoreactive kallikrein along the rat nephron. (submitted for publication).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01237-04 KE

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Hormonal control of transport in kidney epithelial cells in culture

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Michael A. Lang, Ph.D.	Guest Worker	LKEM	NHLBI
Others:	John N. Forrest, Jr., M.D.	Guest Worker	LKEM	NHLBI
	Agnes S. Preston	Chemist	LKEM	NHLBI
	Joseph S. Handler, M.D.	Chief, SMM	LKEM	NHLBI

COOPERATING UNITS (if any)

None

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The response to hormones is being studied in an epithelium as it forms in culture. The epithelium is formed by A6 cells. The hormones to be studied are adenosine, isoproterenol, and vasopressin, agents that act by stimulating adenylate cyclase.

651

PHS-6040
(Rev. 2-81)

Objectives

The study is intended to examine the cellular factors involved in the expression of receptors for hormones that stimulate sodium transport in an epithelium by initially stimulating adenylate cyclase activity. The approach is based on the observation (see last year's annual report) that A6 cells grown in the standard manner (on a petri dish) have adenylate cyclase activity that can be stimulated by guanine nucleotides and by fluoride, but cannot be stimulated by any polypeptide hormone. This is evidence of defective receptor function or defective coupling of hormone receptors to adenylate cyclase. When cells are subcultured from a petri dish to a millipore filter that allows exchange of materials at the basal surface of the epithelium, the epithelium develops responsiveness to several hormones. It should be possible to monitor hormone binding to receptors, activation of adenylate cyclase, and stimulation of sodium transport in epithelia as they develop in culture.

Methods

See previous annual reports for methods of cell culture, for measuring sodium transport, and adenylate cyclase activity. cAMP in cells and medium will be measured by radioimmunoassay. Methods will be developed to quantify specific binding to the epithelium (or a subcellular fraction) of hormones that stimulate cAMP production and sodium transport. Radiolabelled hormones and antagonists suitable for binding studies are available for studying binding of beta adrenergic agents and ribose specific adenosine agonists.

Major Findings

Previous work has demonstrated that A6 epithelia grown on millipore filter bottom cups have a sodium transport response to vasopressin as well as to cAMP, the intracellular mediator of the response to vasopressin. We have now found that the epithelia have a sodium transport response to adrenergic agents and to adenosine. The former response is to beta adrenergic activity in that it is blocked by propranolol and is not blocked by phentolamine. The response to adenosine appears to fit the pattern of a ribose specific adenosine agonist that stimulates adenylate cyclase in that adenosine and 2-chloroadenosine stimulate sodium transport and the stimulatory response is blocked by low concentrations of theophylline.

Proposed Course

1. The development of responsiveness to hormones will be monitored over time (days) as the epithelium develops on a filter bottom cup, by assaying cAMP production and stimulation of sodium transport in response to hormone.
2. Receptor binding (specific binding) of hormones will be assayed and the results correlated with #1.
3. In broken cell preparations, adenylate cyclase activity will be assayed under basal conditions, and in the presence of guanosine triphosphate, and hormones, to evaluate function of the guanine nucleotide binding subunit of the enzyme.

4. Attempts will be made to perturb the normal development using hormones and specific inhibitors that have been shown to alter the phenotypic expression of membrane proteins, in order to gain understanding of the regulation of receptor mediated activation of adenylate cyclase in epithelia.

Publications

Handler, J.S., A.S. Preston, F.M. Perkins, M. Matsumura, J.P. Johnson, and C.O. Watlington: Annals of the New York Academy of Sciences 372:442-454, 1981.

Perkins, F.M. and J.S. Handler: Amer. J. Physiol.:Cell Physiol. 241:C154-C159, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01241-03 KE

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Ionic composition of Necturus gallbladder cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Kenneth R. Spring, DMD, Ph.D.	Physiologist	LKEM	NHLBI
Other:	Richard S. Fisher, Ph.D.	Staff Fellow	LKEM	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Kidney and electrolyte Metabolism

SECTION

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National Heart, Lung, and Blood Institute, Bethesda, MD 20205

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Intracellular ionic activities of potassium and chloride are being measured in Necturus gallbladder epithelial cells undergoing osmotically induced volume changes.

654

PHS-6040
(Rev. 2-81)

Objectives

Necturus gallbladder epithelial cells undergo volume changes when exposed to solutions whose osmolality differs from control solutions. Osmotically induced cell shrinkage or swelling is followed by a rapid readjustment of cell volume back toward the control volume. Such readjustment is termed "volume regulation" and in other tissues involves the flow of solutes into or out of the cell. The objective of these experiments is the identification and quantitation of the ion movements responsible for the observed volume regulation.

Methods

Ion sensitive microelectrodes have been constructed. The electrodes are made from microfiber glass capillaries drawn to a tip of diameter less then 1 μm. The electrodes are siliconized by dipping the tip into trichloromethylsilane for a few seconds and then heating them. The electrodes are stored unfilled until just before use when they are filled from the back end with liquid ion exchange resin. The ion exchangers used in these experiments were sensitive to Cl^- or K^+. Since the ion sensitive electrode has a very high electrical resistance (10^{10} ohms) it exhibits slow response time and great sensitivity to electrical interferences. It is connected to a high impedance electrometer whose output is displayed on a digital voltmeter, interfaced to a small computer. Determination of the end point of electrode drift and slope sensitivity to K^+, or Cl^- is then made objectively by this computer system. A chamber has been designed which permits rapid exchange of the solutions bathing either surface of the gallbladder epithelium.

Major Findings

Intracellular chloride and potassium activities have been determined during changes in the osmolality of the solution bathing the mucosal surface of the Necturus gallbladder. Mucosal hypertonicity, created by the addition of mannitol to the mucosal perfusate, caused an increase in intracellular chloride activity as the cells shrank. The increase was less when the perfusion solutions contained bicarbonate ions. Complete elimination of bathing solution bicarbonate abolished cell volume regulation. Thus (1) cell volume regulation was dependent on the presence of bicarbonate in the bathing solution and (2) the initial transient cell volume decrease following extra cellular hypertonicity was due to water flow out of the cell across the cell membranes without large losses or gains of intracellular ions.

Proposed Course

We will continue to investigate the changes in intracellular ionic composition caused by alterations in solution osmolality and resultant volume regulation. In particular we will measure intracellular sodium activity and attempt to determine whether changes in cell calcium or pH are involved in the response.

Publications

R.S. Fisher, B.-E. Persson and K.R. Spring. Epithelial cell volume regulation: bicarbonate dependence. Science 214:1357-1359, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01243-03

PERIOD COVERED
October 1, 1981 - September 30, 1982

TITLE OF PROJECT (80 characters or less)

D-glucose transport across the renal proximal tubule brush border membrane

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	R. James Turner, Ph.D.	Visiting Associate	LKEM	NHLBI
Others:	A. Moran, Ph.D.	Guest Worker	LKEM	NHLBI
	Janet N. George	Chemist	LKEM	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Kidney and Electrolyte Metabolism

SECTION

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National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	0.50	0.75

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The kinetics, specificity and stoichiometry of D-glucose transport across the renal proximal tubule brush border membrane are being studied in vesicle preparations.

656

PHS-6040
(Rev. 2-81)

Objectives

The continuing goal of this project is to elucidate on both the physiological and molecular levels the mechanism by which D-glucose is reabsorbed from the urine in the proximal tubule. We are presently concentrating on the kinetics, specificity and stoichiometry of transport in isolated brush border membrane vesicles.

Methods

Methods for preparing purified brush border membrane (BBM) vesicles and for carrying out transport and binding studies on them have been discussed in the 1979-80 Annual Report.

Major Findings

In previous Annual Reports (1979-80, 1980-81) we have described two renal BBM preparations, one from the outer cortex (early proximal tubule) and the other from the outer medulla (late proximal tubule). We have also reported that the sodium-dependent D-glucose transport properties of these two preparations are markedly different. More specifically, we observe a low affinity, high capacity transporter in the outer cortical preparation and a high affinity, low capacity transporter in the outer medulla. The ability of various monosaccharides and inhibitors to affect D-glucose flux into the two preparations is also somewhat different indicating differences in specificity. Since the outer cortical and outer medullary preparations contain predominantly BBM from early and late proximal tubules respectively, these results are indicative of D-glucose transport heterogeneity along the length of the proximal tubule.

Over the past year we have gone on to study the glucose transport properties of these two vesicle preparations in more detail in order to better understand the physiological implications of this heterogeneity.

Phlorizin binding: The compound phlorizin is a well-known potent competitive inhibitor of renal D-glucose reabsorption. By simultaneously measuring the $K_{0.5}$ for phlorizin inhibition of D-glucose flux and the $K_{0.5}$ for equilibrium phlorizin binding in each vesicle preparation we have established that the high affinity phlorizin binding site traditionally associated with the BBM D-glucose transporter is in fact associated with the outer cortical and not the outer medullary transport system. The outer medullary transporter is almost two orders of magnitude less sensitive to inhibition by phlorizin than the outer cortical one (the Ki for phlorizin inhibition of D-glucose flux is approximately 1 μm on the outer cortex and 50 μM in the outer medulla at 17°C). We have been unable to identify a phlorizin binding site which can be associated with the outer medullary transporter.

Stoichiometry: We have previously established that the stoichiometry of the outer cortical transporter is 1:1:1 (sodium:glucose:phlorizin). In more recent experiments we have shown that the sodium:glucose stoichiometry of the outer medullary system is approximately 2:1. In order to carry out these measurements we have devised a new method for measuring the activator:substrate stoichiometry for coupled transport systems in vesicle preparations. The procedure, which we refer to as the "static head method", involves searching for the extravesicular to intravesicular activator (sodium) gradient which balances an intravesicular to extravesicular substrate (glucose) gradient. From the known concentration gradients which result in this "static head"

condition the stoichiometry of the transporter can be calculated directly. This procedure has several advantages over the usual method of comparing activatordependent substrate fluxes and substrate-dependent activator fluxes.

Our results on the different sodium:glucose stoichiometries along the nephron have major implications regarding the reabsorptive mechanism for D-glucose. We propose that the functional significance of this arrangement is to enable the kidney to reabsorb glucose from the urine in an energy efficient fashion. The bulk of the glucose load is reabsorbed early in the proximal tubule at an energetic cost of one sodium ion per glucose molecule. Then in the late proximal tubule a larger coupling ratio (2:1) and hence a larger driving force is employed to reabsorb the last traces of glucose from the urine. This arrangement of transporters in series along the proximal tubule leads to a more efficient reabsorptive mechanism for D-glucose than could be achieved by either of the transporters acting alone.

Theoretical Studies: In parallel with our experimental work several theoretical analyses of cotransport models have been carried out in order to provide a better understanding of coupled transport processes in general. We have demonstrated that the general flux equation for the rapid equilibrium carrier model of cotransport can be written entirely in terms of five independent kinetic constants. This result was used in turn to derive a series of rejection criteria for the model.

In order to extend our theoretical studies to steady-state models a computer program has been written which is capable of solving an arbitrarily complex kinetic model.

Proposed Course

Determination of the charge stoichiometry (number of charges transferred per glucose molecule) of the outer cortical and outer medullary transporters is planned. Extension of heterogeneity studies to other substrates is also being considered.

Publications

R.J. Turner and M. Silverman. Interaction of phlorizin and sodium with the renal brush border membrane D-glucose transporter: stoichiometry and order of binding. J. Membrane Biol. 58: 43-55 (1981).

R.J. Turner. Kinetic analysis of a family of cotransport models. Biochim. Biophys. Acta 649, 269-280 (1981)

R.J. Turner. General rate equations and rejection criteria for the rapid equilibrium carrier model of cotransport. Biochim. Biophys. Acta (in press).

R.J. Turner and A. Moran. Heterogeneity of sodium dependent D-glucose transport sites along the proximal tubule: evidence from vesicle studies. Am. J. Physiol. (in press)

R.J. Turner and A. Moran. Stoichiometric studies of the renal outer cortical brush border membrane D-glucose transporter. J. Membrane Biol. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01244-03 KE

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Study of glucose transport by cultured kidney epithelial cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	A. Moran, Ph. D.	Guest Worker	LKEM	NHLBI
Other	R.J. Turner, Ph. D.	Visiting Associate	LKEM	NHLBI
	J.S. Handler, M.D.	Section Chief	LKEM	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Kidney and Electrolyte Metabolism

SECTION
Membrane Metabolism Section

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, Bethesda, MD 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Sugar transport is studied in a cultured epithelium. Transport is assessed by measuring sodium dependent uptake of analogs of glucose into the epithelium as well as into apical membrane vesicles prepared from the cultured epithelium. Conditions are identified that alter the rate of glucose transport.

659

PHS-6040
(Rev. 2-81)

Objective

We and others have demonstrated that epithelia formed in culture by cells of the kidney-derived continuous line designated LLC-PK1 manifest sugar transport like that of the proximal tubule of mammalian kidney. Since the special feature of this transport system is the sodium-coupled glucose transporter in the apical membrane, we began by developing a suitable preparation of apical membrane vesicles for characterizing the transporter (see last year's annual report). The transporter will be studied further. Then, factors that affect the development of the transporter and its function will be examined to gain further understanding of the regulation of expression of differentiated epithelial functions in cultured epithelia as well as further understanding of the glucose transport system in the kidney.

Methods

See last year's report for methods of growing epithelia formed by LLC-PK1 cells in culture, as well as methods for preparing and studying apical membrane vesicles. The ability of cultured epithelia to transport glucose is evaluated by measuring the ability of the epithelium to concentrate a transported, non-metabolized analog of glucose. Radiolabeled tracers are used. The steady state intracellular concentration of alpha methyl glucoside (AMG), a substrate for the carrier, is compared to the steady state intracellular concentration of 3-O-methyl glucoside, a sugar that does not interact with the carrier at the apical plasma mambrane. Both sugars are non-metabolizable. Experiments are performed using epithelia on petri dishes, adding the sugars to the solution bathing the apical surface. Raffinose is used as an extracellular marker.

Major Findings

1. In addition to the vesicle transport findings in last years annual report, a sigmoidal relationship was found between glucose transport rate and the concentration of sodium bathing the vesicles. Analysis of the curve is consistent with a stoichoimetry of two sodium ions per glucose molecule transported. This stoichiometry is supported further by the very high concentrations of glucose the intact epithelia can accumulate (see below). 2. The glucose concentrating capacity of the epithelia increase with time after the cells were initially seeded, reaching a maximum of about 100 (concentration of AMG in cells/concentration in medium) after two to three weeks. Apical membrane vesicles prepared from epithelia at similar stages after seeding show a parallel increase in the rate of sodium dependent transport of AMG, demonstrating the important role of the apical membrane transporter in concentrating glucose in the cell.
3. The concentrating capacity is influenced by the concentration of glucose in the medium in which the cells have been grown. The growth medium (Dulbecco's modified Eagles medium) contains 25mM glucose. When the cells are grown in the same medium containing 5mM glucose, the epithelia develop a five to tenfold greater capacity to concentrate AMG. The effect of growth in high or low glucose medium can be reversed by reversing the glucose concentration of the medium.

Proposed Course

1. We will try to find a defined medium in which the cells will grow so that unknown factors present in serum can be eliminated and the hormone and glucose content of the medium can be exactly determined.
2. We have observed that the cells consume glucose in the medium rapidly, so that the concentration of glucose in the medium falls to levels less than half the nominal (initial) concentration within 24 hours. Therefore, a system will be devised to maintain constant the concentration of glucose as well as other factors in the medium. The effect of the concentration of glucose or other factors in the medium that regulate the development of the transporter can then be studied more precisely.
3. Attempts will be made to grow the cells in medium without substrate for the carrier or with non-metabolized substrates. These media will contain other sugars that have been reported to substitute for glucose in supporting the growth of other cell lines. The purpose of these studies is to test whether the development of the carrier is influenced by transport itself or the resulting cell glucose level.

Publications

A. Moran, J.S. Handler, and R.J. Turner: Na-dependent hexose transport in vesicles from a cultured renal epithelial cell line. Amer. J. Physiol.:Cell (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01245-03 KE

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Amiloride-sensitive volume regulation in Amphiuma red cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Arthur W. Siebens	Biologist	LKEM	NHLBI
Other:	Floyd M. Kregenow, M.D.	Medical Director	LKEM	NHLBI

COOPERATING UNITS (if any)

None

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Laboratory of Kidney and Electrolyte Metabolism

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National Heart, Lung, and Blood Institute, Bethesda, MD 20205

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1.25	1.00	0.25

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Amphiuma red cells incubated in anisotonic media regulate their volume by using specific transport mechanisms for Na and K. The Na uptake mechanism of the response to hypertonicity is blocked by amiloride, but is stimulated following pretreatment with amiloride. Two-thirds of this pretreatment effect can be attributed to amiloride blockage of volume changes; about one-third appears to be due to some other action of the drug.

PHS-6040
(Rev. 2-81)

Objectives

Volume regulation in anisotonic media has been demonstrated in nucleated red cells from a number of species. We have previously shown that Amphiuma erythrocytes return toward their original volume when enlarged in hypotonic media (The Volume Regulatory Decrease or VRD Response) or when shrunken in hypertonic media (The Volume Regulatory Increase or VRI Response). Neither response requires, in the short term, a functional Na-K pump. The recovery phase of the VRD response results from cellular loss of K and (Cl). In the VRI response, cells return toward their original size by gaining Na through an amiloride-sensitive Na-H exchange process. There is also a secondary chloride gain subsequent to these volume regulatory Na movements which takes place through the anion exchange mechanism.

Previous annual reports described a number of similarities between amiloride-sensitive Na transport in the VRI response and that in epithelia. One of the similarities deals with a pretreatment effect of amiloride. If either epithelial cells or shrunken Amphiuma red cells are pretreated with amiloride, Na transport following removal of amiloride exceeds that in untreated controls. We hypothesized that this enhanced Na uptake in shrunken Amphiuma erythroytes had its genesis in amiloride's inhibition of volume changes. This amiloride-pretreatment effect was investigated further this year.

Methods

All methods have been described in previous annual reports.

Major Findings

This year's study has led to the conclusion that only part of the enhanced Na transport following pretreatment with amiloride results from amiloride's inhibition of the volume changes. Approximately one-third of the effect results from some other action of amiloride.

The evidence for this conclusion is as follows: (1) The enhanced Na uptake following amiloride (10^{-3}M) pretreatment is greater than that observed when volume changes are prevented by employing a medium low in Na. Both 10^{-3}M amiloride and low Na treatment are equally effective in blocking volume changes. (2) Pretreatment with a submaximal concentration of amiloride (10^{-5}M) which blocks 70% of the volume regulation produces an enhanced Na uptake rate equivalent to that observed after a pretreatment period with low Na medium. (3) Amiloride pretreatment of cells in isotonic media induces a seven-fold increase in amiloride-sensitive Na uptake despite the absence of detectable amiloride-sensitive Na uptake in isotonic cells not pretreated. No enhanced Na uptake is observed if isotonic cells are pretreated with either a low Na or a normally Na-free medium.

It appears therefore that approximately two-thirds of the increase in Na uptake observed in amiloride-pretreated cells in hypertonic media can be explained by amiloride's ability to block the volume changes. The remaining one-third results from a volume-independent effect of amiloride pretreatment. A similar volume-independent effect of amiloride pretreatment is also presumably responsible for the effect in isotonic cells.

Significance

In the last year there has been a significant increase in the reported number of cation-hydrogen exchange mechanisms operating in cells especially in renal tubules. In addition, Dr. Spring of our laboratory has reported a volume-regulatory amiloride-sensitive Na-H exchange mechanism in Necturus gallbladder which shows many of the characteristics we previously described from the VRI response in Amphiuma red cells. Amphiuma erythrocytes are therefore a useful model system for studying a transport mechanism shared with more complex cells.

The fact that both epithelia and Amphiuma red cells show an amiloride pretreatment effect suggests that this phenomenon is a general one. However, the conclusion reached in epithelial studies, that intracellular Na concentration plays a key role in the pretreatment effect, is not supported by our findings. Rather we favor the view that the amiloride pretreatment effect results mainly from an inhibition of cell volume changes, there being an additional specific effect of amiloride which is independent of its action on changes in volume and intracellular Na concentration.

Proposed Course

We plan to perform studies on the K transport mechanism of the VRD response to determine whether it also operates as an exchanger, transporting K and hydrogen ions in a one to one fashion. This study will be similar to that performed by one of us to demonstrate hydrogen ion coupling to Na in the VRI response. Medium pH will be followed with the red cell anion exchanger blocked to observe whether proton uptake by cells is equivalent to K loss. In addition, protonophores will be used to establish whether hydrogen ions are distributed passively and to determine whether equilibrium cation concentrations are affected by a hydrogen ion gradient when the volume regulatory mechanisms are operating.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01246-02 KE

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Culture and Function Study of Epithelial Kidney Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Maurice B. Burg, M.D.	Chief	LKEM	NHLBI
Others:	Sarah Sariban-Sohraby, M.D.	Guest Worker	LKEM	NHLBI
	Nordica Green, B.S.	Chemist	LKEM	NHLBI
	Joseph Handler, M.D.	Investigator	LKEM	NHLBI
	Roderic Steele, M.D.	Investigator	LTD	NHLBI
	Danielle Chabardes, Ph.D.	Guest Worker	LKEM	NHLBI

COOPERATING UNITS (if any)

Laboratory of Technical Development, NHLBI

LAB/BRANCH
Laboratory of Kidney and Electrolyte Metabolism, NHLBI

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, Bethesda, MD 20205

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2.50	0.25	2.25

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are attempting to establish in culture lines of epithelial cells from defined parts of the nephron.

665

PHS-6040
(Rev. 2-81)

Objective

Study of renal epithelial cells in culture is useful for defining the principles of transepithelial transport, and this is being accomplished in our laboratory with a number of established cell lines. The cell lines that are are being used were initially grown for other purposes, however, and do not express many of the important transport systems of the various parts of the nephron. Even the transporters that are present may not be abundant, which will make it difficult to isolate them and define them chemically. Therefore we are attempting to establish new cell lines from defined tubule segments.

Methods

Our strategy is to dissect fragments of each kind of renal tubule, grow them in culture, and screen the primary cultures as well as any continuous lines for transport function.

Major Findings

Up to now we have concentrated our efforts on the development of methods. Since there are no proceedures known to be generally effective for establishing cell lines that express specific functions, we have been empirically testing a wide variety of conditions that have been reported to be useful for this purpose. The variables that we have been testing include: (1) Media: General formulations, additions of various trace substances, hormones, sera, and defined growth factors. (2) Species: rabbits, rats, mice, toads, and chickens. (3) Surfaces: In addition to conventional plastic culture dishes, also thin collagen membranes, various collagen coatings, and attachment proteins. In general we have been successful in establishing primary growth and demonstrating transepithelial transport in the primaries (see last year's report). However, we have established only a few continuous lines and those do not have any of the transepithelial transport that we are looking for. We find that the cells often die before multiplying to the extent we want or do not survive the splitting that is necessary to establish continuous lines. Therefore, we are testing a variety of techniques for splitting, and we are attempting viral transformations which have been useful for establishing cell lines with other tissues.

Proposed course

We will continue as outlined above, concentrating our immediate efforts on the viral transformations. We will use temperature sensitive mutants of SV40 for rabbit and rat tubules and Rous sarcoma virus for chicken tubules. Temperature sensitive transformation has the theoretical advantage that it may be possible to grow the epithelia at the permissive temperature and study function at the higher non-permissive temperature.

Publications

Burg M, Green N, Sohraby S, Steele R, Handler J: Differentiated function in cultured epithelia derived from thick ascending limbs. Am. J. Physiol. 242:C229-C233, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01247-02 KE

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Urea transport in the kidney

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Mark Knepper, M.D., Ph.D.	Staff Associate	LKEM	NHLBI
Other:	G. Vurek, Ph.D.	Sr. Investigator	LTD	NHLBI

COOPERATING UNITS (if any)

Laboratory of Technical Development, NHLBI

LAB/BRANCH
Laboratory of Kidney and Electrolyte Metabolism, NHLBI

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, Bethesda, MD

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.60	0.50	0.10

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A survey of the urea transport properties of the nephron segments in the kidneys of rats and rabbits is being carried out in order to formulate an integrated model of urea excretion by the kidney. Initial studies were done in rabbit nephrons until it was discovered that nephron segments could be dissected from Sprague-Dawley rats which were specially-maintained to avoid exposure to pathogenic environmental factors. As was true in the rabbit, cortical thick ascending limbs of rats were found to be significantly permeable to urea (apparent permeability: 1.4 x10-5 cm/sec). Preliminary studies in cortical collecting ducts from rats reveals a permeability to urea which is about half that of the cortical thick ascending limbs. Studies of the proximal straight tubules from rabbits did not confirm another laboratory's finding of active urea secretion.

PHS-6040
(Rev. 2-81)

Objectives

The chief long term goal of this project is to understand the role that urea plays in urinary concentration and dilution. An intermediate goal is to determine the extent and mechanism of urea transport in each nephron segment and integrate these data into a model of urea handling by the whole kidney.

Methods

Urea transport in single rat and rabbit nephron segments is being studied using a method for perfusing isolated nephron segments developed in this laboratory. The nephron segments are dissected from New Zealand White rabbits and from Sprague-Dawley rats maintained in a pathogen-free environment. (Dissection of many nephron segments from rats obtained from other sources has proven to be difficult if not impossible presumably because of chronic inflammation due to micro-organisms or other environmental factors.) Urea concentrations are measured in perfused and collected fluid using an ultra-microcolorimeter specially-developed for these studies. The change in the urea concentration between perfused and collected fluid is determined with and without a chemical urea gradient across the epithelium. This allows assessment of permeability properties and active transport capacity of the nephron segment.

Major Findings

The nephron segments are being surveyed on a regional basis. That is, all nephron segments which are physically associated in a given region of the kidney are studied together. The studies done thus far have focused on the segments in the medullary rays of the renal cortex. These rays contain proximal straight tubules, cortical thick ascending limbs, and cortical collecting ducts in a parallel arrangement and are essentially extensions of the outer medulla into the cortex.

Urea Transport in Nephron Segments of the Medullary Rays

	RAT		RABBIT	
	Permeability (x10-5 cm/s)	Active Transport	Permeability (x10-5 cm/s)	Active Transport
Proximal Str. Tubule	-	-	1.5	no
Cortical Thick Ascending	1.4	no	2.0	no
Cortical Collecting Duct	0.7	-	0.2*	-

Dash (-) indicates that measurement has not been made. * Data from radioisotopic measurement: Burg et al. Urea and the Kidney, B. Schmidt-Nielsen, ed., Excerpta Medica, Amsterdam, 1970.

A number of hormones stimulate adenylate cyclase activity in the cortical thick ascending limb of the rat. Separate experiments were done to test whether cyclic AMP affects urea permenability in the rat cortical thick ascending limbs. There was no change in urea permeability or transepithelial potential difference after 10^{-4} M 8-bromo-cAMP was added to the bath.

Significance

Mass balance for water and solutes in the renal medulla reveals that the ability of the kidney to concentrate the final urine depends on the ability of the thick ascending limbs to dilute the tubular fluid. This is accomplished primarily by active NaCl absorption from the water-impermeable thick ascending limb. However, to maximally dilute the tubular fluid, the thick ascending limb must be capable of absorbing other solutes which can contribute significantly to the osmolality of the ascending limb tubular fluid as it emerges from the medulla. This is true particularly for urea which may have a concentration greater than 300 mM near the bends of long loops of Henle according to micropuncture data. The finding of a significant permeability to urea in the cortical thick ascending limbs of rats and rabbits may be important in the generation of a high urinary osmolality. Because of the countercurrent arrangement between cortical thick ascending limbs and proximal straight tubules in the medullary rays, much of the urea absorbed from cortical thick ascending limbs may be passively secreted into the proximal straight tubules and recycled to the medulla. This mechanism may be important in the accumulation of urea in the medulla.

Proposed Course

Measurements of urea permeability in the nephron segments of the medullary rays of rats will be completed. Then, attention will be shifted to the outer medulla, i.e to the permeability properties of the descending limb of Henle's loop, the medullary thick ascending limb and the outer medullary collecting duct. Of these, definition of the permeability properties of the descending limb may be most important, since current theories of concentrating processes in the inner medulla depend on special properties of the descending limb. Finally, attention will shift to the inner medulla, in particular, to the function of the thin ascending limb and inner medullary collecting ducts, both of which are likely to play important roles in the urinary concentrating process. Complete interpretation of these data will depend on mathematical modelling.

Publications

M.A. Knepper. Measurement of osmolality in kidney slices using vapor pressure osmometry. Kidney International 21:653-655, 1982.

G.G. Vurek and M.A. Knepper. A colorimeter for measurement of picomole quantities of urea. Kidney International 21:656-658, 1982.

M.A. Knepper. Urea transport in nephron segments from medullary rays of rabbits: Urea recycling to the renal medulla via the loops of Henle. (submitted for publication).

M.A. Knepper. Urea transport in rat cortical thick ascending limbs in vitro. (abstract). Federation Proceedings 41:x, 1982. (Symposium on the renal concentrating mechanism)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01249-02

PERIOD COVERED
October 1, 1981 - September 30, 1982

TITLE OF PROJECT (80 characters or less)

Isolation and study of brush border membrane transport proteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	R. James Turner, Ph.D.	Visiting Associate	LKEM	NHLBI
Others:	Janet N. George	Chemist	LKEM	NHLBI
	Anne Walter, Ph.D.	Guest Worker	LKEM	NHLBI
	Ellis Kempner	Physicist	LPB	NIADDK

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.15	0.25	0.85

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Isolation of the renal brush border membrane D-glucose transporter is being attempted by conventional protein solubilization and purification techniques and by the development of specific monoclonal antibodies against the transporter. The molecular weight of the transporter has been determined using the technique of radiation inactivation. Labelling of the transporter using sulfhydryl reagents is also being attempted.

671

PHS-6040
(Rev. 2-81)

Objectives

The immediate goal of this project is to develop methods for isolating brush border membrane transport proteins in purified form. Our long term objective is to study these isolated proteins using a number of established biophysical and biochemical techniques in order to gain information about their structure and mechanism of action. Two parallel and complimentary approaches are being employed (a) to attempt to extract and partially purify the transport-related proteins using conventional protein separation, labeling and monitoring methods and (b) to attempt to grow monoclonal antibodies against the proteins of interest. At present we are concentrating on the renal outer cortical brush border membrane (BBM) D-glucose transporter since this protein may be followed throughout extraction procedures by its ability to bind phlorizin.

Methods

Methods for solubilization studies and monoclonal antibody production were described in the 1980-81 Annual Report. All experiments are being carried out using the outer cortical BBM vesicle preparation described in the 1979-80 Annual Report.

Major Findings

(a) Identification of disulfide bonds on the renal outer cortical BBM D-glucose transporter (Turner and George).

We have established that dithiothreitol (DTT), a specific and potent reducing agent for disulfide bonds, has two well defined effects on the outer cortical transporter. The first is a relatively rapid ($T_1/_2$ = 2-3 min) increase in affinity for phlorizin binding while the second is a slower ($T_1/_2$ = 10 min) loss of phlorizin binding and glucose transport activity. The first effect is easily reversed by washing the membranes while the second appears to be irreversible. These results indicate that there are at least two important disulfide bonds on the transporter. Reduction of the first results in a reversible change in affinity while reduction of the second results in irreversible inactivation. Thus the second bond is essential to the functioning of the trans porter.

At present we have confined our studies to the properties of the second (essential) disulfide bond. We have found that this bond can be protected by sugars known to share the transporter as well as by phlorizin. Sodium appears to have no protective ability. Owing to the excellent correlation between the known affinity of various monosaccharides for the transporter and their ability to protect the transporter against inactivation by DTT we conclude that this disulfide bond is closely related to the glucose binding site. The physiological significance of this bond remains to be clarified.

(b) Labelling of the glucose transporter using sulfhydryl reagents (Walter and Turner).

We are attempting to label the transporter making use of the observations discussed above. Our proposed procedure is to incubate membranes with DTT in the presence or absence of protecting sugars, then to tag the sulfhydryl groups resulting from reduction of disulfide bonds with radiolabeled N-ethylmaleimide. Since the degree of labelling of the transporter will be different in protected

and unprotected preparations it should be possible to identify the relevant protein band(s) using polyacrilamide gel electrophoresis. Preliminary experiments to determine appropriate experimental conditions have been carried out and labelling experiments are underway.

(c) Determination of the molecular weight of the glucose transporter by radiation inactivation (Turner and Kempner).

In isolation and reconstitution experiments it is useful to have a reliable measure of the molecular weight of the protein in question. All of the estimations of the molecular weight of the BBM D-glucose transporter in the literature have been indirect, and considerable disagreement now exists. We have determined the molecular weight of the transporter by monitoring the phlorizin binding properties of BBM vesicles as a function of radiation dose. The rate at which an enzyme is inactivated by radiation is proportional to its volume (target size) and hence its mass. Thus molecular weights can be calculated directly from radiation inactivation data using well established empirical relationships. Our results indicate a molecular weight of approximately 110,000 daltons for the outer cortical BBM D-glucose transporter.

Proposed Course

Protein solubilization and purificaton techniques will be attempted in order to obtain fractions enriched in the glucose transporter. Proteins identified by sulfhydryl group labelling will be used to produce monoclonal antibodies to the transporter.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01250-02 KE

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Acidification and Bicarbonate Transport by Renal Tubules

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	James Atkins, M.D.,Ph.D.	Staff Fellow	LKEM NHLBI
Other:	Maurice B. Burg, M.D.	Chief	LKEM NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	1.00	0.25

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Maximal transepithelial pH gradient is determined in isolated perfused collecting ducts from rat kidneys, comparing cortical, outermedullary and innermedullary segments.

674

PHS-6040
(Rev. 2-81)

Objectives

The pH of the urine is an important factor controlling net acid excretion by the kidney. Final adjustment of the urine pH occurs in the collecting duct system. Previous studies in this laboratory have examined bicarbonate reabsorption and steady state pH in the proximal tubules and in the cortical collecting duct of the rabbit. These factors in vivo determine the buffer load to the medulla and the more distal segments. Since it is thought that the distal segments can only reabsorb a relatively small buffer load , buffer load is an important determinant of final urine pH. The present study is measuring the transport characteristics of the most distal tubule segments. Specifically the study is attempting to measure the maximal transepthelial pH gradient that these tubules can maintain and the factors that influence this gradient.

Methods

Collecting ducts are dissected from 100g rats without the use of collagenase, obtaining segments from the cortex, the inner stripe of the outer medulla or the white medulla within one or two mm of the papillary tip. The tubules are perfused as previously described. pH of the collected fluid is measured in the collecting pipette using a micro glass pH electrode. The design of this pipette system is adequate to measure pH without artifact from diffusional loss of CO_2 before the measurement. pH is determined at slow perfusion rates and the limiting value is approximated as the average of values obtained with perfusates of lower and higher pH. The Bath pH is measured with a similar electrode, and the maximal transepithelial pH gradient is determined. The effects of various changes in the bathing solutions are examined.

Major Findings

This is the first in vitro study showing acidification by the rat collecting ducts. Bath pH has been maintained at 7.4. Most segments have been examined with a bath containing 25 mM bicarbonate and with a bath that was normally bicarbonate free.

Part of collecting duct	Steady State pH with bicarb	without bicarb
Cortical	7.2	6.0
Outer medullary	6.5	
Inner medullary	7.2	6.8

These findings are quite similar to the proximal tubule and cortical collecting duct of the rabbit. The findings suggest that bicarbonate back-leak may limit the transepithelial pH gradient. The steady state pH of the inner medullary collecting duct of the rat is appreciably higher than the urine pH. This suggests that the conditions in vivo differ from those in vitro in some critical, but yet undetermined way.

Proposed Course

Further studies of bicarbonate transport rate, as well as pH, will be carried out to define the characteristics of the various rat collecting systems. Various factors are being examined that might maximize the transepithelial gradient in the inner medullary collecting duct.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01251-02 KE

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

The application of nuclear Magnetic resonance to the study of cellular physiology

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Robert S. Balaban, Ph.D.	Staff Fellow	LKEM	NHLBI
Other:	Jack Orloff, M.D.	Dir., IR		NHLBI
	Sarah Sohraby, M.D.	Guest Worker	LKEM	NHLBI
	Richard Knazek, M.D.	Investigator	LPP	NCI
	Mark Knepper, M.D., Ph.D.	Investigator	LKEM	NHLBI

COOPERATING UNITS (if any)

Laboratory of Pathophysiology

LAB/BRANCH
Laboratory of Kidney and Electrolyte Metabolism

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INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.00	0.25	0.75

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Nuclear magnetic resonance (NMR) spectroscopy is capable of non-invasive on-line determinations of intracellular concentrations and turnover of numerous metabolites as well as inorganic ions. We have been applying this technique to the study of cellular physiology both in vitro and in vivo. Studies have been performed on the effect hypoxia and hypovolemic shock on the relative concentrations of high energy phosphates in the in vivo rat brain and kidney. The 14N resonances from the in vivo rat kidney, brain, liver and leg rat have also been characterized. In addition, we have made progress in combining NMR with tissue culture preparations, in order to use the advantages of a well defined and controlled cell population to investigate cellular physiology using this powerful technique.

677

Objectives

The objectives of the project have been; (1) to characterize the relationship between the ^{31}P detectable intracellular high energy phosphate compounds and the physiological state of cells in vivo, (2) to characterize the ^{14}N resonances detected in vivo in order to access the use of this nuclide as a probe, and (3) to adapt the NMR and cell culture technologies so that a stable cell culture system can be studied by NMR under physiological conditions with good signal to noise and reasonable time resolution.

Methods

All of the in vivo studies were performed on anesthetized animals in a specially designed animal cradle for the NMR spectrometer magnet. During these experiments the animal were ventilated and blood pressure constantly monitored. The radio receiving and transmitting coil was a flat 2 turn 1.0 cm in diameter coil for ^{31}P or a 6 turn, 1.5 cm in diameter coil for ^{14}N. All experiments were performed in an 8 cm bore magnet at 147 mHz for ^{31}P, 26 mHz for ^{14}N and 360 mHz for ^{1}H. The magnetic field was shimmed using the ^{1}H signal from the tissue water.

The NMR studies on cultured cells use cells either in suspension or attached to hollow perfused fibers. Cells in suspension are being studied using techniques published previously (1). The attached cell studies have been performed in collaboration with Drs. Richard Knazek and Sarah Sohraby using the perfused fiber system developed by Dr. Knazek. The cells are grown on the outside of perfused hollow fibers which are packed in a glass cylinder. This yields a sufficiently high concentration of cells to obtain reasonable signal to noise for ^{31}P NMR determination of intracellular metabolites. We have sucessfully adapted an NMR probe to accomodate the perfusion lines and the chamber containing the perfused fibers and growing cells.

Major Findings

We have found that the high energy phosphate componds can be monitored by ^{31}P NMR in the brain and kidney of a rat during experimental perturbations. Abundant ATP and creatine phosphate (CrP) were detected in the brain, the amounts of free inorganic phophate and sugar phosphates were low. ATP, but no CrP was detected in the kidney. Inorganic phosphate and sugar phosphates were higher in the kidney than in brain. In addition, a resonance peak from glycerol-phosphorylcholine was detected from the papilla of the kidney. The experimental perturbation we used were either a graded hypovolemic shock, induced by venous bleeding, or hypoxia, induced by lowering the oxygen content of the ventilatory gas. In both protocols the kidney ATP levels were found to decrease before those of the brain suggesting that this tissue is more sensitive. In both tissues the blood pressure or the oxygen tension had to be decreased at least 50% to detect a change in ATP (kidney) or ATP and CrP (brain). These data suggest that the steady state levels of ATP and CrP may not be very sensitive indicators of the state of these organs since they are so highly regulated that they not change significantly until extreme conditions are reached. ^{14}N NMR spectra from the in vivo rat brain, liver, kidney and leg were collected. Resonances from free ammonia, choline-glycerol phosphoryl choline,

betaine, and urea were clearly resolved. However, most amino acids lack sufficient chemical shift differences to resolve them individually. The ^{14}N NMR spectra indicated that the kidney and liver contained very high concentrations of choline and related compounds (>10 mM), while these compounds were much lower concentrations in the brain and leg. From these studies it was clear that ^{14}N NMR may be useful in investigating choline and ammonia metabolism in vivo.

With the cell culture system we have sucessfully initiated studies on cell suspensions of HTCBH cells and human lymphocytes, investigating both intracellular pH and energy metabolism. A6 cells have been grown in the perfused fiber system and ^{31}P NMR spectra have been collected. More work is required, however, to find the optimal type of fiber for growing cells.

Proposed Course

From these studies, and others, it is apparent that steady state levels of high energy phosphate compounds are not very sensitive to subtile changes in the physiological state of the tissue. Skeletal muscle is a possible exception. Therefore, more information on the regulation of these compounds maybe found by investigating their steady-state turnover by 2D NMR or saturation transfer techniques. The appropriate in vitro experimentation and hardware modifications are now underway to allow us to perform these types of experiments in vivo. The NMR studies on cultured cells will focus on the study of energy metabolism of these cells to investigate the coupling of active ion movements with energy metabolism. Specifically, the interaction between aerobic glycolysis, active Na extrusion and proton-lactate efflux will be studied. Further trials with other fiber compositions and other configurations to grow attached cells are forseen to maximize cell growth at the required density for NMR investigations.

(1) Balaban, Gadian, Radda, and Wong. 1981. Anal. Biochem. 116:450-455.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01255-01 KE

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Vasopressin-stimulated permeability to water in cultured renal epithelial cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	J.N. Forrest, Jr., M.D.	Guest Worker	LKEM	NHLBI
Others:	A.S. Preston	Chemist	LKEM	NHLBI
	A. Moran, Ph. D.	Guest Worker	LKEM	NHLBI
	R. Steele, Ph. D.	Investigator	LTD	NHLBI
	K. Spring, D.M.D., Ph. D.	Physiologist	LKEM	NHLBI
	J.S. Handler, M.D.	Chief, SMM	LKEM	NHLBI

COOPERATING UNITS (if any)

Laboratory of Technical Development, NHLBI

LAB/BRANCH
Laboratory of Kidney and Electrolyte Metabolism

SECTION
Section on Membrane Metabolism

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.75	0.50	1.25

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Methods are being developed to assay transepithelial permeability to water in epithelia in culture. Epithelia formed in culture by kidney derived continuous lines of epithelial cells and by primary cultures of kidney cells will be evaluated for a water permeability response to vasopressin and to cAMP.

680

PHS-6040
(Rev. 2-81)

Objectives

Although vasopressin has several effects on the kidney, the major effect is increased permeability to water in collecting ducts, which results in reabsorption of water from the urine (conservation of water). Previous work with epithelial cultures of cells derived from vasopressin responsive tissues has failed to demonstrate a water permeability response, although related functions such as receptors for vasopressin, adenylate cyclase stimulated by vasopressin, and cAMP mediated stimulation of sodium transport and of permeability to urea have been identified. Other evidence of the cellular equipment for the water permeability response such as cytosolic vacuoles containing particle aggregates have also not been found. It is the purpose of this study to improve methods for assaying water permeability of cultured epithelia and to develop primary and/or continuous cultures of epithelial cells that manifest a water permeability response to vasopressin or its intracellular mediator, cAMP. Such cultures will be invaluable in gaining further understanding of the cellular events in the water permeability response.

Methods

Water permeability may be assessed by three methods.

1. The diffusional permeability to THO. Preliminary experiments indicate that the epithelium must cover an area of 5 square cm in order to detect the anticipated flux of labelled water. Our standard filter bottom cups should be suitable.
2. Measurement of volume flow along an osmotic gradient across the epithelium. A flux chamber capable of measuring flow rates as low as 3 microliters/hour/10 square mm area with a minimal detectable leak of less than one microliter/hour is being developed.
3. A chamber developed by Dr. K. Spring of this laboratory will be used to estimate changes in cell volume . When the cultured epithelia are bathed with a hypotonic solution on the apical surface and vasopressin or cAMP is added to the basal surface, the cell should swell as the hormone increases water permeability.

Cultures will be prepared from toad urinary bladder and from mammalian renal medulla. Epithelial cells from toad urinary bladder will be cultured (see previous annual reports) and culture conditions varied to promote the expression of the water permeability response. Epithelial cells from the renal medulla of mouse and rat will be cultured as described by others who have found that the cultures make cAMP in response to vasopressin. These cultures will be tested for epithelial functions of interest.

Major Findings

Epithelioid cultures have been prepared from rat renal medulla. When grown on collagen membranes, these cultures have a transepithelial potential difference of about one millivolt, apical surface negative. The response to vasopressin has not been tested.

Proposed Course

Methods for assessing water permeability will be developed further. Methods for culturing the epithelia will be modified.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01256-01 KE

PERIOD COVERED
October 1, 1982 to September 30, 1983

TITLE OF PROJECT (80 characters or less)

Lipid protein interactions in rabbit renal proximal tubule brush border membrane vesicles.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Y. Fukuhara, M.D., Ph.D.	Visiting Fellow	LKEM	NHLBI
Other:	R.J. Turner, Ph.D.	Visiting Associate	LKEM	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Kidney and Electrolyte Metabolism

SECTION
Membrane Metabolism Section

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.15	1.00	0.15

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Lipid protein interactions in rabbit renal brush border membrane vesicles are being studied by determining the temperature at which various intrinsic proteins experience a local lipid phase transition. The proteins being studied are the enzyme alkaline phosphatase, the sodium dependent D-glucose transporter and the ionophore valinomycin.

PHS-6040
(Rev. 2-81)

Objectives

The purpose of this project is to test the suggestion recently put forward by Smedt and Kinne (Biochim. Biophys. Acta 648:247-253, 1981) that temperature-dependent alterations in brush border membrane protein-lipid interactions may be relatively unspecific and affect a number of proteins simulaneously.

Methods

Methods for preparing purified outer cortical brush border membrane (BBM) vesicles and for carrying out transport and binding studies on them have been discussed in the 1979-80 Annual Report.

Major Findings

We have measured the alkaline phosphatase and phlorizin binding activities of BBM vesicles as a function of temperature over the range 4-37°C. In addition we have investigated the temperature dependence of the mobility of the carrier protein valinomycin over the range 8-28°C. The latter studies were carried out by monitoring the ability of valinomycin to induce a membrane potential in the presence of an intravesicular to extravesicular potassium gradient. Sodium-dependent glucose transport was used to monitor the presence of the membrane potential. When plotted on an Arrhenius plot (log activity vs. log temperature), each of these proteins displays a break in the slope of the graph at different temperatures. The break in the Arrhenius plot is indicative of a change in the activation energy of the process under investigation, which for membrane localized proteins is indicative of a local change in membrane fluidity (phase transition). The breaks in the Arrhenius plots occur at 12-14°C for alkaline phosphatase, 15-16°C for valinomycin and 25-26°C for phlorizin binding. These results clearly indicate that these proteins exhibit markedly different lipid environments in contradiction to the suggestion of Smedt and Kinne.

Proposed Course

The practicality of using temperature studies to investigate the thermodynamic properties of membrane localized proteins will be investigated.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01257-01 KE

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Ammonia production by individual nephron segments of rat kidney

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	David Good, Ph.D.	Guest Worker	LKEM	NHLBI
Other:	Maurice Burg, M.D.	Chief	LKEM	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	1.00	0.25

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Ammonia production by individual nephron segments of rat kidney is being measured using a newly developed microfluorometric assay for ammonium ion. These studies are designed to assess which tubule segments are able to synthesize ammonia and to determine which segments alter their production rate in response to metabolic acidosis. Although all segments tested from normal animals appear to be capable of synthesizing ammonia, metabolic acidosis stimulates production only in proximal tubule.

685

Objective

Ammonia synthesized in renal cells is the major urinary buffer for excretion of acid in rat, dog and man. Renal ammonia production is increased during metabolic acidosis and decreased in metabolic alkalosis. The purpose of the present studies is to evaluate which nephron segments are capable of producing ammonia and to determine which segments alter their production rate in response to changes in systemic acid-base balance.

Methods

New methods for assessment of ammonia production by single nephron segments have been developed. Tubule segments dissected from collagenase treated rat kidneys are incubated under oil in microliter fluid droplets of known composition. Nanoliter samples are removed from the fluid droplets at 10 minute intervals and assayed for ammonia. Time-dependent increases in droplet ammonia concentration permit calculation of ammonia production rate per unit tubule length.

Ammonia in nanoliter samples is measured by reductive amination of alpha-ketoglutarate: alpha-ketoglutarate + NH_4^+ + NADH $\xrightarrow[\text{dehydrogenase}]{\text{glutamate}}$ glutamate + NAD. NADH oxidation, which is proportional to sample ammonia concentration, is quantitated in a newly developed microfluorimeter.

Major Findings

1. In normal rats ammonia is produced by all nephron segments tested. Production rate is two to five times greater in proximal tubule segments than in segments of thick ascending limb or collecting duct.
2. In rats made chronically acidotic by pretreatment with ammonium chloride, ammonia production is increased only in segments of proximal tubule.
3. A microfluorometric method was developed for measurement of ammonia in nanoliter volumes. The method can reliably detect as little as one picomole of ammonia.

Significance

Incubation of specific nephron segments in vitro is ideal for study of systemic regulation of ammonia production and for evaluation of biochemical pathways involved in ammonia synthesis. In addition, development of a sensitive assay for ammonia will allow characteristics of ammonia transport in different nephron segments to be studied by in vitro microperfusion techniques.

Proposed Course

1. Incubation of tubule segments in vitro will be used to identify systemic factors that contribute to the regulation of ammonia production that occurs in proximal tubule segments in response to changes in acid-base status.
2. In vitro microperfusion techniques will be used to compare ammonia permeability and transport characteristics in different nephron segments. This information is essential to understanding the intra-renal mechanisms that contribute to regulation of systemic acid-base balance.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01258-01 KE

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Corrolating of structure and function during transport in Amphiuma erythrocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Floyd M. Kregenow, M.D.	Medical Director	LKEM	NHLBI
Others:	Sarah Beygu	Guest Worker	LKEM	NHLBI
	Marie Cassidy, M.D.	Investigator	GWSM	
	Ino Ifrim	Biologist	GWSM	

COOPERATING UNITS (if any)

George Washington School of Medicine, Department of Physiology

LAB/BRANCH
Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION
National Institutes of Health, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.00	0.75	0.25

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Amphiuma erythrocytes contain an extensive cytoplasmic tubulovesicular system which may be the site of hydrogen ion formation prior to transport of the hydrogen out of the cell during the volume regulatory increase response. This cell is much more complicated than previously thought, containing several other structures which also may be involved in the volume regulatory responses.

688

PHS-6040
(Rev. 2-81)

Objectives

Many vertebrate cells after having first been shrunken or enlarged osmotically can correct their size while continuing to incubate in anisotonic media. To accomplish this adaptive response, these cells activate otherwise quiescent transport mechanisms that control total salt content. These mechanisms were first characterized in this laboratory using nucleated erythrocytes from duck and the salamander, Amphiuma means. Their similarity to transport proceses in epithelia has led to the hypothesis that like or similar mechanisms judiciously placed in an epithelial cell, are responsible for all or part of transepithelial salt movements.

Especially interesting are those studies in Amphiuma red cells dealing with the mechanism activated by shrinkage and responsible for the cell's subsequent enlargement. In this instance the fundamental transport event involves the one to one exchange of osmotically active extracellular Na for osmotically inactive intracellular hydrogen ions. Cells enlarge by gaining Na and water and become more alkaline in the process.

The plasma membane has been considered the site at which all relevant events related to these transport proceses take place, the Amphiuma erythrocyte notwithstanding. This assumption is bolstered by present anatomical evidence which pictures non-mammalian erythrocytes, like mammalian erythrocytes, as simply a bag of hemoglobin limited only by the plasma membrane and containing as an aside, a dormant nucleus, a few mitochondria and a marginal band of microtubules, considered structural supports for the cell's ellipsoid shape.

This year's project began by addressing this supposition, albeit indirectly. We tried to follow the formation and loss of intracellular hydrogen ions in Amphiuma erythrocytes during the enlargement process. It was hoped that the cell's large size would make such an approach feasible. If the genesis of all transport related events is the plasma membrane then we expected to observe an intracellular pH gradient originating at the plasma membrane during transport.

Methods

In addition to the methods described in previous annual reports, we used the standard technique of fluorescence and Nomarski light microscopy as well as those of scanning and transmission electron microscopy.

Findings

Initially we simply looked for evidence of an intracellular pH gradient by examining transporting cells in the presence of various pH sensitive dyes. Fluorescent and non-fluorescent dyes were used. Of those dyes scrutinized, acridine orange proved most informative. Rabin, Chang and Sachs (Biochem. 17:750-753, 1978) have shown that in the intracellular canaliculi of parietal cells and in vesicles prepared from the canaliculi, acridine orange is a useful pH indicator fluorescing orange-red in the acid environment of the intraluminal space. This capacity of the dye depends upon its ability to bind and stack to properly spaced negative sites that develop in response to an imposed pH gradient across the membrane.

Unexpectedly acridine orange made visible an extensive tubulovesicular system in the cytoplasm of control cells. This system fluoresces orange-red provided the pH of the intraluminal space is more acid than the cytoplasm. Consequently maximal fluorescence and visualization is obtained when the cytoplasmic pH is raised by alkalinizing the medium. Under certain experimental conditions this system can be observed with Nomarski optics. Visualization under these conditions has permitted a three-dimensional graphic analysis. These studies along with electron microscopic studies, mentioned later, suggest that this intracellular system is analogous to the smooth endoplasmic reticulum.

Several findings suggest the tubulovesicular system is the site at which the hydrogen ions involved in the transport process accumulate. First, placing cells in a hypertonic medium initiates transport and simultaneously produces an increase in the orange-red fluorescence which as mentioned signifies a decrease in the pH within the tubulovesicular system. More hypertonic solutions cause this change in fluorescence and transport to increase in parallel. Second, the increase in fluorescence and transport show roughly the same time course; both decrease once the cells have approached their original volume. Finally, the most intense fluorescence is seen under the same unusual experimental conditions in which we find the transport rate to be maximal. That is after washing away amiloride, a specific inhibitor of the transport process, from cells which had preincubated in a hypertonic solution containing amiloride.

All the light microscopy studies mentioned above have been performed in collaboration with Mr. Larry Ostby of the Department of Medicine Photography. The electron microscopic examination of cells was performed by one of us under the tutelage of Ms. Ina Ifrim in Dr. Marie Cassidy's laboratory. Ultra-thin sections and several modifications in the standard staining techniques make the visualization of the tubulovesicular system possible. The canaliculi of the system are tortuous and extend throughout the cytoplasm from the nucleus to the plasma membrane often in juxtaposition to mitochondria. Other structures are also present. Amphiuma erythrocytes possess an extensive Golgi apparatus, lysosomes, multivesicular bodies and a microtubular system composed of thousands of tubules rather than a few reported previously. Preliminary studies indicate that many of these structures change their morphology during transport, raising the possibility that a dynamic equilibrium exists between the membranous elements of the cell during transport.

Gentle homogenization produces a vesicular preparation of these cytoplasmic membranous elements which is relatively free of both nucleus and plasma membrane. After cell rupture the plasma membrane collapses on the nucleus and the two separate together upon differential centrifugation. This vesicle preparation shows a time-dependent Na uptake.

Proposed Course

These new-found structures in the Amphiuma erythrocyte will be studied further for their possible involvement in volume regulation.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01259-01 KE

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

The cotransport pathway in erythrocytes from patients with Bartter's syndrome

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	Jeff Korff, M.D.	Investigator	HED	NHLBI
Others:	John Gill, M.D.	Investigator	HED	NHLBI
	Floyd Kregenow, M.D.	Medical Director	LKEM	NHLBI

COOPERATING UNITS (if any)

Hypertension and Endocrine Division, NHLBI, NIH

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Laboratory of Kidney and Electrolyte Metabolism

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The erythrocytes from patients with Bartter's syndrome show an elevated intracellular Na and an increase in Na efflux through both the pump and cotransport pathways. Only the increase in the cotransport pathway may be a primary feature of the disease.

691

PHS-6040
(Rev. 2-81)

Objective

In ouabain-treated avian erythrocytes, a Na-K-Cl cotransport system modulates changes in cell volume by causing net ion movements. Osmotically obligated water movements accompany alterations in cell salt content and determine the changes in size. Under certain experimental conditions, this transport process can cause Na to leave the cell against its electrochemical gradient. A distinguishing feature of this system is its sensitivity to inhibition by "loop" diuretics such as furosemide and bumetanide.

Ouabain-treated human red cells also possess a transport process, which, again, under certain restrictive experimental conditions can cause Na to leave the cell against its electrochemical gradient. This Na efflux pathway is part of a transport system, like that in avian erythrocytes, that requires the presence of Na, K and Cl and is inhibited by furosemide.

A linked Na-K-Cl cotransport mechanism which is insensitive to ouabain but inhibited by "loop" diuretics also exists in a variety of "leaky" epithelia and has been postulated to be responsible for Cl reabsorption in the mammalian thick ascending limb of Henle.

Patients with Bartter's syndrome do not reabsorb chloride from the kidney normally. Clearance studies have implicated the thick ascending limb as the site of this transport defect. Furthermore, defective Cl reabsorption in the thick ascending limb is sufficient to explain the pathogenesis of all of the abnormalities described in the syndrome.

Our objective in this study was to compare the rate of ouabain-resistant, furosemide-sensitive Na efflux in erythrocytes from patients with Bartter's syndrome and a group of normal control patients. We chose this component of Na efflux as a way of assessing a presumably general Na-K-Cl cotransport system and hoped to observe differences in the two groups of erythrocytes.

Methods

The methods have been described in previous annual reports.

Major Findings

The enclosed table shows the means and standard deviations for cell water content, intracellular Na and K concentrations, ^{22}Na efflux rate constants and ^{22}Na efflux for five patients with Bartter's syndrome and 22 normal controls. Erythrocytes from patients with Bartter's syndrome show significant differences in four parameters. They have an elevated intracellular Na concentration and show an augmented total ^{22}Na efflux; this increase in total ^{22}Na efflux is a consequence of accelerated loss through both the pump (A. ouabain-sensitive component) and the cotransport system (B. ouabain-resistant furosemide sensitive component).

	Bartter's Syndrome (n) (5)	Normal (22)
Na (mEq/L RBC)	9.2 ± 1.4	7.1 ± 1.3
K (mEq/L RBC)	98.8 ± 4.8	101.9 ± 4.2
H_2O (%)	62.9 ± 1.4	63.9 ± 0.97
Total efflux		
rate constant (hr^{-1})	0.424 ± 0.074	0.371 ± 0.060
efflux (mEq/L RBC/hr	3.86 ± 0.30	2.61 ± 0.64
A. ouabain-sensitive		
rate constant	0.274 ± 0.061	0.256 ± 0.046
efflux	2.48 ± 0.154	1.80 ± 0.23
B. oubain-resistant, furosemide sensitive		
rate constant	0.068 ± 0.022	0.046 ± 0.019
efflux	0.63 ± 0.22	0.32 ± 0.12
C. oubain and furosemide resistant		
rate constant	0.082 ± 0.029	0.069 ± 0.017
efflux	0.75 ± 0.24	0.49 ± 0.13

The increase in erythrocyte Na may result from the patients hypokalemia and metabolic alkalosis, two clinical features of the syndrome. Chronic exposure to low K plasma concentrations and/or a high HCO_3 concentration may depress pump activity sufficiently to cause the cellular Na concentration to rise slightly in vivo. The accelerated pump activity may simply be, in turn, a consequence of the elevated intracellular Na concentration and a renewed effort on the part of the pump to lower its Na concentration once it has been brought in contact again with a solution whose composition is more normal.

The only difference of importance then is the ^{22}Na loss through the cotransport pathway which can probably not be explained entirely by the increase in intracellular Na. Note that the increase in intracellular Na and rate constant resulted in a nearly doubling of ^{22}Na loss through this pathway.

Proposed Course

Future studies will reexamine these parameters in the two groups of erythrocytes, using red cells from patients with Bartter's syndrome which have had their intracellular Na concentration normalized. Although erythrocyte Na concentration can be normalized by several means, the initial attempt will simply involve converting the patient's hypokalemia and metabolic alkalosis with sufficient doses of oral KCl and NaCl to maintain normal plasma electrolyte values. Since the cotransport system mediates Na movement both in and out of the cell, future studies will also involve Na influx and net determinations in the presence and absence of furosemide. Finally, to establish whether a circulating plasma factor is responsible for the observed abnormalities, red blood cells from both groups will be incubated in their own and each other's plasma.

Publiations

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01260-01 KE

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

The cotransport pathway in erythrocytes from patients with essential hypertension

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Jeff Korff, M.D.	Visiting Scientist	HED	NHLBI
Others:	John Gill, M.D.	Investigator	HED	NHLBI
	Floyd M. Kregenow, M.D.	Medical Director	LKEM	NHLBI

COOPERATING UNITS (if any)

Hypertension and Endocrine Division, NHLBI

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National Institutes of Health, Bethesda, MD 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We find no difference in the rate of ^{22}Na loss through the cotransport pathway of erythrocytes from patients with essential hypertension, regardless of whether the hypertension is the salt-sensitive or the non-salt sensitive type.

695

PHS-6040
(Rev. 2-81)

Objectives

Essential hypertension is a disease of unknown etiology. One hypothesis proposes that these patients have a defective NaCl transport mechanism in the smooth muscle of their blood vessels. The findings of Garay and Tosteson that a Na-K and a Na-Li cotransport pathway was altered in the erythrocytes of family members and patients with essential hypertension was therefore noteworthy. It was hoped this abnormality was a manifestation of the hypothetical defective transport process which was present in this instance in a cell system more amenable to study than smooth muscle. Recently the usefulness of these red cell transport studies to the study of hypertension has been challenged by several laboratories. These conflicting claims may result from differences in the study populations since essential hypertension is a heterogeneous disease. Patients with essential hypertension can be divided into salt-sensitive (SS) and non-salt sensitive groups (nSS) on the basis of their blood pressure response to dietary manipulation. Our objective was to characterize Na efflux through the cotransport pathway in erythrocytes from a group of hypertensive patients who have been so characterized.

Methods

The response of hypertensive patients to controlled dietary salt-intake during a three week inpatient hospitalization served to classify them as salt-sensitive or non-salt sensitive. After having had all medications discontinued three weeks prior to hospitalization, patients were placed on an isocaloric diet containing 109 mEq Na for one week, 9 mEq of Na for the second week, and 249 mEq of Na for the third week. Patients whose average mean BP on day seven, of the high sodium diet (third week) exceeded by 8% or more their average mean BP on day seven of the low Na diet (second week) were classified as salt-sensitive. Patients who had either no increase in BP or an increase of 3% or less were classified as non-salt sensitive. Blood was drawn for the transport studies between the third and seventh day of the first week when dietary Na intake was normal. The method used to measure ^{22}Na efflux and extracellular Na have been described in previous annual reports.

The mean and standard deviations for Na concentration and Na efflux for erythrocytes from the two groups of hypertensive patients and a group of control subjects with normal bp are presented in the following table. The total ^{22}Na efflux has been separated into three components based on responsiveness to 10^{-4} M furosemide.

Major Findings

Patients (n)	[Na] mEq/L RBC	Ouabain-sentitive	Ouabain-resistant; furosemide-sensitive	Ouabain and furosemide resistant
SS (8)	7.2 ± 1.5	1.9 ± .26	.41 ± .16	.57 ± .14
nSS (8)	7.6 ± 2.0	2.0 ± .34	.40 ± .11	.58 ± .09
Control (22)	7.1 ± 1.3	1.8 ± .23	.32 ± .12	.49 ± .13

The blood of hypertensive patients and controls showed no significant difference in the Na transport rate through the furosemide-sensitive cotransport system (column 3). There are also no differences in erythrocyte intracellular Na concentration.

Proposed Course

Since red cells from salt-sensitive, non-salt sensitive hypertensive and control subjects showed no differences, these studies will not be pursued further.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01261-01 KE

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Intracellular pH regulation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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Others:	Anne Walter, Ph.D.	Guest Worker	LKEM	NHBLI
	John Bader, M.D.	Res. Microbiol.	LTVG	NCI

COOPERATING UNITS (if any)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The mechanism of intracellular pH regulation will be elucidated for a variety of glycolyzing cell types including epithelial cell lines, transformed cell lines and renal tubule segments such as the collecting duct. Cell metabolism, especially lactate production from glycolysis, is the major acid load of these cells. Thus, mechanisms for lactate and proton efflux will be analyzed and related to the cells' metabolic status. The plasma membrane fluxes of protons and lactate and their electrochemical gradients will be measured to characterize the transport processes as active or passive. The carrier(s) involved will be characterized in terms of ion requirements, functional chemical sites, and competitive and noncompetitive interaction with inhibitors.

698

Objectives

The first objective of this study is to develop techniques for studying intracellular pH regulation in conjunction with the metabolic status of the cell. The cells being examined initially are glycolytic cells, either epithelial or transformed. Ehrlich ascites tumor cells and cells derived directly by collagenased treatment of tissue (e.g. renal distal tubules) will be used. Predictably, the major source of acid production in these cells is lactic acid. To learn how lactic acid is eliminated from the cells, the gradients for lactate and hydrogen ion across the cell membrane under various ambient and metabolic conditions will be determined. Secondly, the flux rates of hydrogen ion and lactate must be determined. Finally the mechanism(s) for proton and lactate efflux and influx will be characterized with respect to cofactors, inhibitors, voltage sensitivity, and coupling. To complete this last step, a ghost or vesicle preparation of the cell membrane will be developed so that transport mechanisms can be seperated from metabolic effects.

Methods

Most experiments are being performed on cell suspensions at 37°C in a chamber designed for several simultaneous measurements. The chamber consists of a water-jacketed glass cylinder with appropriate ports for oxygen, pH and other ion specific electrodes. In addition two optical windows permit simultaneous optical spectroscopic or fluorometric monitoring of the suspension. A very sensitive rapid scanning spectrophotometer provides the means to observe the time course of absorbance shifts in the relatively turbid cell suspensions by compensating for light scattering. The initial measurements are being performed on two readily available cell types, the transformed cell line, HTCBH, and Ehrlich ascites tumor cells. Intracellular pH will be determined in three ways: (1) from the internal inorganic phosphate shift as observed by ^{31}P NMR; (2) from absorbance changes of the pH-sensitive cytoplasmic dye, 6-carboxyfluoroscein; and (3) by intracellular pH electrodes. Other cytoplasmic constituents which are not detectable by ^{31}P NMR or ^{1}H NMR are measured conventionally after cells have been separated from their extracellular fluids by rapid centrifugation through an oil layer into perchloric acid. For example, intracellular lactate and pyruvate levels are measured enzymatically in the perchlorate digest. The efflux of hydrogen ion equivalents is monitored by a pH stat. Lactic acid efflux is measured optically from the rate of cytochrome-c reduction coupled to lactate through yeast lactate dehydrogenase in the extracellular fluid. This method is noninvasive, allows measurement of lactate production simultaneously with other measurements, and will provide data on initial rates of efflux. Membrane potential is determined by an intracellular electrode. The transport process is being characterized in terms of ion requirements and sensitivity to known inhibitors of anion and cation transport. Specific inhibitors of lactate efflux are being sought.

Major Findings

At present the pH stat, pH, electrode and oxygen electode have been interfaced with the computer and an appropriate chamber designed. Hydrogen ion equivalent efflux (determined by pH stat), is equal to the sum of lactic acid plus acid from respiration. The rate is about 25 nmol/mg-protein-min in the

presence of glucose. The rate of proton efflux decreases as external pH decreases and external lactate concentration increases. It is not clear whether this is an effect on production (i.e. glycolysis) or efflux of lactate. Preliminary ^{31}P NMR observations of HTCBH cells indicate that the internal inorganic phosphate signal is adequate to measure intracelluar pH as well as monitor the intracellular levels of ATP, ADP and the sugar phosphates. The cells can be rapidly separated from their extracellular fluid by spinning through silicon oil with only 0.8% contamination from the bathing fluid. Thus, necessary chemical determinations of cell constituents, e.g. lactate and sodium, are being made. Several putative lactate transport inhibitors have been tested. Some appear to be effective and will be pursued further. The properties of the HTCBH cells and Ehrlich ascites tumor cells appear to be identical.

Significance

Internal pH regulation is a fundmental problem facing all cells. Cells in culture and many cells of the body (e.g. kidney medulla, muscle, retina) are known to produce large quantitites of lactic acid and consequently must have a mechanism capable of removing this acid load efficiently. Description of the mechanism may enhance our understanding of lactate and other monocarboxylic acid transport in normal cells and/or may identify a unique feature of transformed or malignant cells.

Proposed Course

The methods that we have been developing are all now working. We will first study the electrochemical gradients and fluxes of protons and lactate under a variety of steady-state conditions in HTCBH and Ehrlich ascites cells. Then, the stoichiometry of the efflux of the two ions will be determined from rapid kinetic experiments. Finally, the characterization of the transport mechanisms, including ion requirements, inhibitors, and functional groups will be pursued. The approaches and understanding developed will then be applied to other cell types.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01262-01 KE

PERIOD COVERED
October 1, 1981 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Non-invasive studies of enzyme catalyzed reaction rates state by two dimensional NMR spectroscopy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Robert S. Balaban, Ph.D.	Staff Fellow	LKEM	NHLBI
Others:	James A. Ferretti, Ph.D.	Investigator	DCRT	NIH

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The reaction rate constants of enzyme catalyzed reactions are being determined under steady-state conditions by non-invasive 2D NMR spectroscopic techniques. The 2D NMR technique permits the simultaneous determination of all of the rate constants involved in a reaction sequence, and explicitlly displays them in the form of a 2D plot. In addition, the 2D NMR experiment also provides information on the relative size of the substrate pools involved in the reaction, critical information for determining reaction rates in the compartmentalized cell cytosol. To date, three enzyme systems have been studied; phosphoglucose isomerase, adenylate kinase, and creatine kinase. In all three cases, unique information on both the mechanism and flux charateristics of the reactions have been obtained. Due to the non-invasive nature of the technique, it should be readily applicable to the determination of enzyme reaction rates within intact cells in vitro as well as in vivo.

701

PHS-6040
(Rev. 2-81)

Objectives

The determination of enzymatic rate constants aids our understanding of both the mechanism and control of enzymatic processes. The objective of this project was to investigate the feasiblity of using the recently developed techniques of 2D NMR to measure enzyme catalyzed reaction rates.

Methods

All experiments were performed on ^{31}P at 147 mHz using a 8 cm bore Nicolette NMR spectrometer. The 2D NMR technique used was basically that described by Ernst and co-workers (1) for simple chemical exchange reactions. We have modified these procedures to study more complex enzyme catalyzed reactions. Basically, kinetic 2D NMR measurements use the inherent frequency label of atomic nuclei to determine the rate of conversion of one molecular species into another. Since most ^{31}P nuclei in phosphate compounds involved in energy metabolism of the cell (i.e. AMP, ADP, PO_4, ATP, sugar phosphates, etc.) have distinctly difference resonance frequencies, this technique should be applicable to them. The three enzymes we have begun to study are; phosphoglucose isomerase, adenylate kinase, and creatine kinase. All enzymes were studied at equilibrium. The reaction rates and concentrations of the substrates were determined from the ^{31}P NMR resonance of the interconverting molecular species.

Major Findings

The 2D NMR technique proved capable of measuring most of the rate constants involved in the three enzymes studied. In addition, we demonstrated that the size of the exchanging substrate pools could also be determined in a model two compartment system. Specifically we found that phosphoglucose isomerase catalyzes not only the isomerization of glucose-6-phosphate to fructose-6-phosphate, but also the anomerization of glucose-6-phosphate between its alpha and beta forms. The alpha anomer of glucose-6-phosphate was found to react rapidly in the isomerization reaction, while the isomerization of the beta form, under equilibrium conditions, was extremely slow and may occur only after the anomerization of beta glucose-6-phosphate to alpha glucose-6-phosphate. For the adenylate kinase reaction we found that the exchange rate between the alpha ADP and AMP phosphate nuclei was approximately 6 times faster than the exchange rate between the beta ADP and gamma ATP phosphate nuclei. This implies that the ADP to AMP and the ADP to ATP reactions can reversibly occur independently and that an enzyme-phosphate complex must be formed during both reactions to maintain stoichiometry. These data demonstrate that the unidirectional fluxes through these enzyme systems can be determined by 2D NMR. After further charaterization in the in vitro enzyme systems, we will then be able to perform and interpret 2D NMR experiments in intact cells.

Proposed Course

To continue to study the flux characteristics of the major energy metabolism enzymes in vitro. Then use this information to begin studies on enzymes in intact organelles, such as mitochondria, and finally in intact cells and animals. The overall goal of these future studies will be to determine the rate relationships between the different enzyme systems involved in energy metabolism and

specifically characterize whether they are operating near equilibrium (i.e. unidirectional fluxes greater than net flux) or under near irreversible conditions (i.e. unidirection flux equals the net flux).

Ref. (Jeener, Meier, Bachmann and Ernst. 1979. J. Chem. Phys. 17: pp 4546-4553)

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01263-01 KE

PERIOD COVERED
October 1, 1981 - September 30, 1983

TITLE OF PROJECT (80 characters or less)

Na-K-ATPase efficiency in Tumerogenic cells.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Robert S. Balaban, Ph. D.	Staff Fellow	LKEM	NHLBI
Other:	John P. Bader, M.D.	Investigator	LTVG	NCI

COOPERATING UNITS (if any)

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SUMMARY OF WORK (200 words or less - underline keywords)

The efficiency of Na-K-ATPase in intact red blood cells, renal cells, several amphibian epithelial tissues and in vitro enzyme preparations is approximately 2 K+ pumped per ATP hydrolyzed. In contrast, studies on the reconstituted Na-K- ATPase from transformed cells have indicated that this enzyme is inefficient in these cells (i.e. approximately 0.2 K+ pumped/ATP hydrolyzed) and has been speculated to be the source of ADP and phosphate driving glycolysis at a high rate in transformed cells. We have studied the efficiency of Na-K-ATPase in intact transformed cells and found that the enzyme pumps approximately 2K+/ATP or has the same efficiency as in normal cells. In conclusion, we find no evidence, in the intact cell, that Na-K-ATPase is inefficient in transformed cells and that it is unlikely that the inefficient turnover of this pump is responsible for the ehanced lactate production of these cells.

704

PHS-6040
(Rev. 2-81)

Objectives

The primary objective of this investigation was the determination of the efficiency of Na-K-ATPase in intact transformed cells. The efficiency of Na-K-ATPase was estimated from the ouabain sensitive instantaneous rate of K influx upon the addition of K to K-depleted cells and the associated increase in oxygen consumption. The K/ATP ratio was calculated by assuming 6 or 5.6 ATP were produced for every oxygen molecule consumed.

Methods

Three cell lines were used in this study: Ehrlich ascites, cells and hamster and chicken embryo cells transformed with Rous sarcoma virus. Cell suspensions were K^+ depleted by washing the cells 5 times in K^+-free medium with 10 to 20 minute incubations at 5 or 37°C between each wash. The K^+ influx was measured by the disappearance of K from the extracellular space with a K^+ selective electrode (Orion). Oxygen consumption was simulataneously determined with a Clark type oxygen electrode. The chamber itself consisted of a water jacketed glass cylinder, with pH and oxygen electrodes and an injection port mounted in the sides of the cylinder. The K^+ electrode was inserted from the top, sealing the chamber.

Major Findings

The K^+/ATP ratio for the ouabain sensitive K influx into these three transformed cell lines was approximately two. These data indicate that Na-K-ATPase has the same efficiency in these intact transformed cells as it does in normal cell types. Thus, the notion that Na-K-ATPase is inefficient and driving metabolism at a high rate in the transformed cell is apparently incorrect.

Proposed Course

One of the original observations which led to the hypothesis that the pump was inefficient in transformed cells was that lactate production was extremely sensitive to ouabain, an inhibitor of Na-K-ATPase. We have found that glycolysis is closely linked to Na-K-ATPase turnover in these transformed cells. We are now investigating the possible mechanisms by which this phenomenon may occur.

Publications

R.S. Balaban and J.P. Bader: Na-K-ATPase is not inefficient in the RousSarcoma transformed HTC-BH cell. Biophysical J. 37(408a), 1982.

R.S. Balaban and J.P. Bader: The Efficiency of Na-K-ATPase in tumorigenic cells. Nature (submitted).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01264-01 KE

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Apical sodium uptake in cultured kidney cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Sarah Sariban-Sohraby, MD	Visiting Fellow	LKEM	NHLBI
Other:	Maurice B. Burg,	Chief	LKEM	NHLBI
	R. J. Turner, Ph.D.	Visiting Associate	LKEM	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Kidney and Electrolute Metabolism

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, Bethesda, MD 20205 We studied the

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.75	0.50	0.25

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We studied the apical sodium uptake in a continuous line of epithelial cells derived from toad kidney; this uptake is stimulated by aldosterone and completely inhibited by amiloride the ki of amiloride is 5x10(-7) M and the transporter is saturable with a km approximately 15 mm.

706

PHS-6040
(Rev. 2-81)

Objective

The goal of this research is to characterize the sodium transporter in a tight epithelium (toad kidney epithelial cells in culture) by studying its kinetics and hormonal regulation.

Methods

The cells are grown on a cup-like filter-bottomed structure designed so that the cells are fed both by their apical and basal sides and that transepithelial voltage and short-circuit current can be measured prior to the uptake experiments. For the uptake studies, ^{22}Na is applied to the apical side of the cells only, along with an extracellular marker; the reaction is then stopped with icecold stop solution and the intracellular content of radioactive sodium is counted.

Major Findings

Apical sodium influx is completely inhibited by amiloride at the concentration of 10^{-4} M. The Ki of amiloride is 5×10^{-7} M. After incubation of the cells with aldosterone, the influx increases three times. The peak of stimulation is observed after 6 hours. The sodium transporter is saturable with a Km ~ 15 mM. These data indicate that sodium enters the cells through amiloride sensitive channels and that aldosterone increases the permeability of the apical membrane. Looking at the sodium uptake at various luminal Na and amiloride concentrations we showed that Na and amiloride compete for the occupancy of the transporter.

Proposed Course

Knowing the kinetics of the sodium transporter in cultured toad kidney cells, we plan to make plasma membrane vesicles from the same cells so as to be able to study the transporter in conditions where intra and extra-vesicular contents are controlled. The goal is to eventually isolate and reconstitute the transport protein.

Publications

None

Annual Report of Laboratory of Molecular Cardiology
National Heart, Lung and Blood Institute
October 1, 1981 to September 30, 1982

The Laboratory of Molecular Cardiology is investigating the regulation of contractile proteins in smooth muscle and non-muscle cells such as platelets. In addition studies have been initiated to characterize the cardiac cell surface molecules responsible for regulating ionic conductance in the heart and to investigate the role of anti-myocardial cell surface anitbodies in the pathogenesis of human hypertrophic cardiomyopathy.

Purification of Myosin Light Chain Phosphatases (M.D. Pato)

Four different phosphatase activities have been purified from smooth muscle. Phosphatase I is a trimer which dephosphorylates and thereby activates the enzyme myosin kinase. The catalytic subunit of phosphatase I dephosphorylates myosin, converting it to a form that cannot be activated (i.e. the MgATPase activity) by actin. Phosphatase II is a Mg-dependent enzyme resembling a number of other phosphatases in both structure (M.W. = 43,000), cation dependence and substrate specificity. Phosphatase III, which can be separated from phosphatase I by hydrophobic chromatography, appears to be substrate specific for myosin. Phosphatase IV, which can dephosphorylate myosin and the isolated 20,000 dalton light chain of myosin, may be a proteolytic product of phosphatase III. Phosphatase I and II have been characterized with respect to their structure, cation requirements, inhibitors and substrate requirements.

Regulation of the Actin-Activated MgATPase Activity of Myosin by Phosphorylation: (J.R. Sellers)

Using the soluble two-headed fragment of smooth muscle myosin, heavy meromyosin (HMM), it was demonstrated that phosphorylation of the 20,000 dalton light chain proceeds in a random (i.e. non-cooperative) manner. In contrast, dephosphorylation, using two different phosphatases proceeds in a positive cooperative manner. Both heads of heavy meromyosin must be phosphorylated in order for actin to activate the MgATPase activity of myosin.

Unphosphorylated HMM was found to bind to actin with only a slightly weaker affinity (4-fold) than phosphorylated HMM, in the presence of ATP. Since we previously showed that unphosphorylated HMM, in contrast to phosphorylate HMM, cannot be activated by actin, the binding data indicates that phosphorylation does not act to unblock the active site on myosin. It implies that some step in the kinetic cycle, other than the binding of HMM-nucleotide to actin, is regulated by phosphorylation.

Myosin Phosphorylation in Intact Smooth Muscle : (P. de Lanerolle)

The state of phosphorylation of the 20,000 dalton light chain of myosin has been studied in intact tracheal muscle during contraction and relaxation. Myosin is phosphorylated when tracheal muscles are contracted with methacholine, and the extent of phosphorylation and tension generated are dependent on the concentration of methacholine. Forscilin, an agent that increases the intracellular concentration of cAMP, causes smooth muscle relaxation and a concomitant decrease in light chain phosphorylation. The antibody raised to myosin kinase has been used to rapidly purify myosin kinase from a number of sources and we are using this antibody to monitor the state of myosin kinase phosphorylation following smooth muscle contraction and relaxation.

Smooth Muscle and Human Platelet Myosin Kinase: (M. Nishikawa)

The sites phosphorylated and effect of phosphorylating myosin kinase from smooth muscle and human platelets are being studied. cGMP-dependent protein kinase and C-kinase, as well as a number of other kinases will be used in this experiment. The phosphorylation of myosin kinase will be carried out in the presence and absence of bound calmodulin and the results obtained will be compared to those previously obtained using cAMP-dependent protein kinase.

Studies on Three Functional Domains of Myosin Kinase: (M.E. Payne)

A brief tryptic digestion of smooth muscle myosin kinase removes the two sites that can be phosphorylated by cAMP-dependent protein kinase. These sites are contained in a 27,000 dalton peptide. The remaining peptide of approximately 80,000 daltons contains the calmodulin binding site, as well as the site responsible for the transfer of phosphate. A more extensive digestion results in the loss of Ca-calmodulin dependence of myosin kinase, but not of the transferase activity. Utilizing a number of techniques we plan to study the spatial and functional relation of various sites in smooth muscle myosin kinase.

Purification of Myosin Light Chains by HPLC: (M.A. Movsesian)

A technique for the rapid purification and determination of the state of phosphorylation of myosin light chains is being developed. This technique should provide mg quantities of purified myosin light chain from various sources. These light chains will be used in producing affinity purified antibodies and in exchanging light chains on various myosins.

Anti-myocardial Cell Surface Antibodies in the Pathogenesis of Human Hypertrophic Cardiomyopathy.: (M.D. Schneider)

Patients with hypertrophic cardiomypathy (HCM) were screened for circulating anti-β-1-receptor autoantibodies. Eight of 18 sera tested resulted in 20-70% precipitation of solubilized turkey erythrocytes beta-1-receptors. The prevalence of the anti-β-1-receptor autoantibodies in HCM and control populations and characterization of the antibody-receptor interaction will be studied.

Characterization of Cardiac Cell Surface Proteins Regulating Ionic Conductance in the Heart: (M.D. Schneider)

The biochemical basis and ionic mechanism underlying the succession of electrophysiologic behavior during myocardial differentiation is being studied. Apamin, an antagonist of the calcium-dependent potassium channel will be used as a molecular probe for pacemaker development. For example, it will be utilized for affinity chromatography in an effort to purify the various channel antigenic domains in order to elucidate "pacemaker" differentiation and function.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01665-07 MC

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Smooth Muscle and Human Platelet Myosin Light Chain Kinase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	Masa Nishikawa	Visiting Fellow	MC NHLBI
Other:	R.S. Adelstein	Chief, Laboratory of Molecular Cardiology	MC NHLBI
	Wm. Anderson, Jr.	Chemist	MC NHLBI
	J. Maurice Miles	Biological Laboratory Technician	MC NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Molecular Cardiology

SECTION

INSTITUTE AND LOCATION
NHLBI - NIH Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	1.2	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A number of different kinases, such as cGMP-dependent protein kinase, and C-kinase will be used to phosphorylate myosin light chain kinase isolated from platelets and bovine trachea. The site(s) phosphorylated by the various kinases will be identified following phosphorylation of myosin kinase in the presence and absence of bound calmodulin. The effect of phosphorylation on myosin kinase activity and on the ability of myosin kinase to bind calmodulin before and after phosphorylation will be studied.

711

Project Description:

The regulation of the enzyme myosin light chain kinase by covalent phosphorylation is being studied using myosin kinase isolated from human platelets and bovine trachea.

Human platelet myosin kinase has been purified to homogeneity, and similar to smooth muscle myosin kinase was found to incorporate 2 moles of phosphate when cAMP-dependent protein kinase was used to phosphorylate it, in the absence of bound calmodulin . When calmodulin was bound to myosin kinase during the phosphorylation step, only one mole of phosphate was incorporated. Previously we have shown (Hathaway D.R. et al Nature 291: 252-254, 1981) that the introduction of 2 moles of phosphate results in a decrease in platelet myosin kinase activity, when activity is measured in the presence of a non-saturating concentrations of calmodulin.

Myosin light chain kinase has also been purified from bovine tracheal smooth muscle. The molecular weight of this kinase, 160,000 dalton, is significantly larger than turkey gizzard myosin kinase (130,000 daltons). The trachea myosin kinase was found to cross-react with the affinity purified antibody raised to myosin kinase.

In addition to serving as a substrate for cAMP-dependent protein kinase, tracheal myosin kinase could also be phosphorylated by cGMP-dependent protein kinase. (This enzyme was a gift of Dr. Thomas Lincoln, University of South Carolina School of Medicine). cGMP-dependent protein kinase incorporated 1 mole of phosphate/mole of myosin kinase when calmodulin was not bound and 0 moles when calmodulin was bound to myosin kinase.

We plan to study the ability of trachea and platelet myosin kinase to serve as a substrate for a number of different protein kinases, such as cGMP-dependent protein kinase and C-kinase. The effect of phosphorylation on myosin kinase activity, as well as the sites phosphorylated in myosin kinase, by these various enzymes, will be characterized.

Publications:

Adelstein, R.S.: Calcium and calmodulin in Kyoto. Nature (News & Views) 294: 693-694, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01752-04 MC

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Purification of Myosin Light Chain Phosphatases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	Mary D. Pato	Visiting Associate	MC NHLBI
Others:	R.S. Adelstein	Chief, Laboratory of Molecular Cardiology	MC NHLBI
	Wm. Anderson, Jr.	Chemist	MC NHLBI
	Estelle Harvey	Biologist	MC NHLBI
	J. Maurice Miles	Biological Laboratory Technician	MC NHLBI

COOPERATING UNITS (if any)
Dr. Shmuel Shaltiel Fogarty Scholar

LAB/BRANCH
Laboratory of Molecular Cardiology

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.4	0.8	0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Four different phosphatase activities were isolated from turkey gizzard smooth muscle. Phosphatase I is a trimer which dephosphorylates myosin light chain kinase, resulting in activation of this enzyme. The catalytic subunit of phosphatase I, but not the holoenzyme, can dephosphorylate myosin, resulting in inhibition of the actin-activated MgATPase activity. Phosphatase II is a Mg^{2+}-dependent enzyme, which resembles a number of other phosphatases purified from other sources, such as cardiac muscle. Phosphatase III, which can be separated from phosphatase I by hydrophobic chromatography, is active in dephosphorylating myosin and appears to be substrate specific. Phosphatase IV, which dephosphorylates both myosin and the isolated light chain of myosin may be a product of phosphatase III.

PHS-6040
(Rev. 2-81)

Project Description: Myosins isolated from smooth muscle and non-muscle cells require phosphorylation of their 20,000 dalton light chain in order to undergo actin-activation of their MgATPase activity. The enzyme that catalyzes the phosphorylation, myosin kinase, is a substrate of cAMP-dependent protein kinase. Incorporation of two moles of phosphate into the myosin kinase descreaes its activity.

Two different phosphatases have been purified to apparent homogeneity from turkey gizzards myosin kinase, and have been characterized: Phosphatase I is a trimer composed of three polypeptide chains (60,000, 55,000 and 38,000). The 38,000 dalton polypeptide chain is the active catalytic subunit and has been separated from the 60,000 and 55,000 dalton subunits. Both the catalytic subunit and the holoenzyme are inhibited by divalent cations, nucleotides and pyrophosphate. The holoenzyme dephosphorylates diphosphorylated myosin kinase, removing two moles of phosphate from the enzyme when calmodulin is not bound to myosin kinase. When calmodulin is bound, phosphatse I dephosphorylates only site 2 on myosin kinase. This site is thought to be the site that interacts with the calmodulin binding site.

In addition to phosphatase I, phosphatase II, a Mg^{2+}-dependent phosphatase, has been purified and characterized. This enzyme has a molecular weight of 43,000 and is inhibited by nucleotides, pryophosphate and NaF. It is similar in its physical and biochemical properties to phosphatases purified from canine heart and rat liver.

Phosphatase III, an enzyme that dephosphorylates smooth muscle myosin, appears to differ from the catalytic subunit of phosphatase I in two important properties: it does not dephosphorylate the isolated light chain of myosin and its activity is not inhibited by divalent cations. Phosphatase IV elutes as a separate peak from phosphatase III following Sephacryl S-200 gel filtration. When phosphatase III is stored in the cold for 1 or 2 days and then filtered through Sephacryl S-200 its K_{av} increases to that of phosphatase IV, suggesting that IV, may be a product of III.

Publications:

Adelstein, R.S., Pato, M.D., Sellers, J.R., de Lanerolle, P. and Conti, M.A.: Regulation of actin-myosin interaction by reversible phosphorylation of myosin and myosin kinase. Cold Spring Harbor Symposium and Quantitative Biology, XLVI: 921-928. 1982.

Pato, M.D., Sellers, J.R., Conti, M.A. and Adelstein, R.S.: Reversible phosphorylation of smooth muscle myosin kinase. In Smooth Muscle Contraction. (Stephen, N. ed.). Manitoba, Canada, Marcel Dekker, Inc. (In press) 1982.

Pato, M.D. and Adelstein, R.S.: Purification of smooth muscle phosphatases. Methods in Enzymology, 85: 308-315. 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01785-03 MC

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Myosin Phosphorylation in Intact Smooth Muscle

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	Primal de Lanerolle	Staff Fellow	MC NHLBI
Others:	R.S. Adelstein	Chief,	MC NHLBI
	Wm. Anderson, Jr.	Chemist	MC NHLBI
	J. Maurice Miles	Biological Laboratory Technician	MC NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Molecular Cardiology

SECTION

INSTITUTE AND LOCATION
NHLBI - NIH Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	1.2	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have studied myosin phosphorylation during contraction of tracheal smooth muscle (TSM). We have found that myosin is phosphorylated when thse muscles are contracted with methacholine and that the extent of phosphorylation and the tension generated are dependent on the concentration of methocholine used to contract TSM.

We have also studied myosin dephosphorylation and cAMP formation during relaxation of TSM. The data indicate that myosin dephosphorylation, but not cAMP formation, is essential for relaxation of TSM.

715

PHS-6040
(Rev. 2-81)

Myosin phosphorylation appears to play an important role in the process of excitation-contraction coupling in smooth muscle. Biochemical experiments have demonstrated that phosphorylation by a calcium-calmodulin-dependent kinase stimulates the actin-activated Mg^{++}-ATPase activity of smooth muscle myosin. These data form the basis of the hypothesis that myosin phosphorylation regulates smooth muscle contraction. Physiological experiments have provided support for this hypothesis by demonstrating that myosin is phosphorylated during contraction and dephosphorylated during relaxation of intact smooth muscle. However, these physiological experiments were performed on muscles stimulated with a single agonist concentration and little information is available regarding myosin phosphorylation in response to stimulation with various agonist concentrations. Since the dose-response relationships for phosphorylation and tension should be similar if myosin phosphorylation does, in fact, regulate smooth muscle contraction, we have studied myosin phosphorylation in tracheal smooth muscle stimulated with a broad range of concentrations of the cholinergic agonist, methacholine.

Tracheal smooth muscle strips, separated from both the adventitium and the mucosa, were mounted in myobaths and connected to tension transducers. Following an equilibration period, muscles were stimulated with various concentrations of methacholine. Muscles were frozen with clamps cooled in liquid nitrogen and assayed for myosin phosphate content using an immunoprecipitation-isoelectric focusing procedure. Data from these experiments indicate the following: (a) Myosin is rapidly phosphorylated when tracheal muscles are contracted with a maximal or half-maximal concentration of methacholine; (b) Myosin phosphorylation precedes the generation of maximal tension; (c) Both maximal isometric tension and the steady-state level of myosin phosphorylation are dependent on the agonist concentration used to stimulate tracheal smooth muscle. These data are consistent with the hypothesis that myosin phosphorylation regulates smooth muscle contraction but indicate a relatively complex relationship between myosin phosphorylation and the generation of isometric tension.

We have also been studying the relationships among changes in cAMP levels, myosin dephosphorylation and relaxation of tracheal smooth muscle. These experiments are predicated on the observation that phosphorylation of myosin kinase by cAMP-dependent protein kinase leads to a decrease in myosin kinase activity, *in vitro*. Tracheal smooth muscle strips were contracted with 10^{-6}M methacholine and then relaxed by adding either 4×10^{-7}M atropine or 4×10^{-5}M forscolin. Muscles were frozen at various times during relaxation and assayed for myosin phosphate content and cAMP levels. The data from these experiments demonstrate that cAMP levels are elevated and that myosin is dephosphorylated as the muscles relax following the addition of forscolin. On the other hand, myosin is dephosphorylated and muscles relax, without an increase in cAMP levels, following the addition of atropine. These data suggest the presence of at least two different mechanisms (one that involves an increase in cAMP and one that does not) for relaxating tracheal smooth muscles. They also suggest that myosin dephosphorylation may be an essential event in the relaxation process of tracheal smooth muscle.

We are now studying myosin kinase phosphorylation in order to determine whether an increase in the level of cAMP can be correlated with phosphorylation of myosin kinase.

Publications:

de Lanerolle, P., Condit, J., Tannenbaum, M. and Adelstein, R.S.: Myosin Phosphorylation, Agonist Concentration and Contraction of Tracheal Smooth Muscle. Nature, in press 1982.

Adelstein, R.S., de Lanerolle, P., Sellers, J.R., Pato, M.D. and Conti, M.A.: Regulation of contractile proteins in smooth muscle and platelets by calmodulin and cyclic AMP. In Recent Advances in Ca^{2+} and Cell Function, Calmodulin and Intracellular Ca^{2+}-Receptors. Kyoto, Japan pp. 313-331, 1982.

Adelstein, R.S., Pato, M.D., Sellers, J.R., de Lanerolle, P. and Conti, M.A.: The regulation of contractile proteins by reversible phosphorylation of myosin and myosin kinase. Basic Biology of Muscles. A Comparative Approach. B.M. Twarog, R.J.C., Levine & M.M. Dewey ed. Raven Press, N.Y. pp. 273-281, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01786-03 MC

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Role of Phosphorylation as a Regulatory Mechanism in Muscle Contraction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	James R. Sellers	Staff Fellow	MC NHLBI
Others:	R.S. Adelstein	Chief, Laboratory of Molecular Cardiology	MC NHLBI
	Wm. Anderson, Jr.	Chemist	MC NHLBI
	J. Maurice Miles	Biological Laboratory	

COOPERATING UNITS (if any)
Evan Eisenberg and Lois Greene, NHLBI, Laboratory of Cell Biology

LAB/BRANCH
Laboratory of Molecular Cardiology

SECTION

INSTITUTE AND LOCATION
NHLBI - NIH Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	1.2	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The mechanism of phosphorylation-dependent myosin-linked regulation is being investigated using myosin and its subfragments from turkey gizzard. This problem is being attacked using a variety of steady state and transient kinetic techniques. Recent experiments have shown that although the MgATPase activity of unphosphorylated myosin cannot be activated by actin, actin can in fact, bind to this form of myosin.

718

PHS-6040
(Rev. 2-81)

We are interested in determining the mechanism by which phosphorylation of the 20,000 dalton light chain of smooth muscle myosin regulates the actin-activated MgATPase activity . To study this we have utilized the soluble two-headed subfragment heavy meromyosin (HMM).

We have examined the correlation between extent of phosphorylation and the actin-activated MgATPase activity and have found that phosphorylation of the two heads is random and that both heads must be phosphorylated before the MgATPase activity of either head can be activated by actin. This results in a non linear plot of % ATPase vs % phosphorylation. In contrast, when the correlation between dephosphorylation of fully phosphorylated HMM and the MgATPase activity is examined, a purely linear relationship results. One explanation for this linear relationship is that the dephosphorylation of HMM by the phosphatases occurs in a positive cooperative manner.

In another series of experiments the binding constant of both phosphorylated and unphosphorylated HMM to actin was measured in the presence of ATP. There was only a four fold difference in the binding constants under conditions where there was a 25 fold larger V_{max} for the actin activated MgATPase activity for phosphorylated HMM. These results rule out any model where the unphosphorylated light chain sterically or allosterically blocks the interaction of actin and HMM and implies that some step in the kinetic cycle other than binding of HMM nucleotides to actin is the step which is regulated by phosphorylation.(These experiments were carried out with Dr. Evan Eisenberg)

In collaboration with Drs. Lois Greene and Evan Eisenberg we have made a detailed study of the ionic strength dependence of the binding of the single headed subfragment (S-1) from smooth muscle to actin in both the absence and in the presence of AMPPNP and ATP. These results were compared to results obtained by ourselves and others using S-1 from rabbit skeletal muscle. At low ionic strength (0.012M) smooth muscle S-1 binds more weakly to actin than does skeletal S-1 in both the presence and absence of nucleotides. In contrast, as the ionic strength is raised there is only a small weakening of the binding constant of smooth muscle S-1 to actin whereas that of skeletal S-1 is dramatically weakened. Similarly the effect of ionic strength on the actin-activated MgATPase activity of smooth muscle S-1 is much less than on skeletal muscle S-1 MgATPase activity. This indicates that at physiological ionic strengths (0.1M) the binding of smooth muscle S-1 to actin is considerably stronger than that on skeletal S-1. These results may explain the high tension values which have been reported in intact smooth muscle cells.

Publications:

Sellers, J.R., Pato, M.D. and Adelstein, R.S.: Reversible phosphorylation of smooth muscle myosin, heavy meromyosin and platelet myosin. J. Biol Chem. 256: 13137-13142. 1981.

Schier, J.J. and Adelstein, R.S.: Structural and enzymatic comparison of human cardiac muscle isolated from infants, adults and patients with hypertrophic cardiomyopathy. J. Clin. Invest. 69: 816-825. 1982.

Adelstein, R.S., Sellers, J.R., Conti, M.A., Pato, M.D. and de Lanerolle, P. (1982) Regulation of Smooth Muscle Protein by Calmodulin and Cyclic AMP Fed Proc. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 04201-01 MC

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Purification of Contractile Proteins by HPLC

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	Matthew A. Movsesian	Medical Staff Fellow	MC NHLBI
Others:	R.S. Adelstein, M.D.	Chief, Laboratory of Molecular Cardiology	MC NHLBI
	Wm. Anderson, Jr.	Chemist	MC NHLBI
	J. Maurice Miles	Biological Laboratory Technician	MC NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Molecular Cardiology

SECTION

INSTITUTE AND LOCATION
NHLBI - NIH Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	1.2	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are studying mechanisms of contractility associated with smooth and cardiac muscle myosin light chains. Using reverse phase HPLC, we have purified the 20,000 MW phosphorylatable light chain from turkey gizzard, and plan to use the technique to purify light chains from a number of different sources. We plan to develop an assay for myosin light chain phosphorylation in cardiac muscle treated with various inotropes. Finally, we are assaying in vitro inhibition of myosin kinase activity by vasodilators.

721

PHS-6040
(Rev. 2-81)

Our research in smooth and cardiac muscle biochemistry follows three principal lines of investigation: 1. Mechanism of action of vasodilators in smooth muscle relaxation, 2. application of reverse phase high performance liquid chromatography (HPLC) to the purification and characterization of contractile proteins, and 3. use of HPLC to examine the biochemical changes associated with inotropy.

1. Mechanism of action of vasodilators

It has been observed that vasodilators which block the influx of Ca^{++} across plasma membranes may exert their smooth muscle relaxant effects through intracellular actions as well. One Ca^{++}-antagonist, felodipine, has been shown in a preliminary study to bind to calmodulin. This raises the possibility that felodipine may inhibit the ability of calmodulin to activate myosin light chain kinase by a mechanism similar to that of the tricyclic antidepressants.

We are studying the ability of various vasodilators, including the Ca^{++} antagonist drugs, to inhibit the phosphorylation of smooth muscle myosin light chains by Ca^{++}/calmodulin-activated myosin kinase (in vitro). To date, nitroprusside and the Ca^{++} antagonist nifedipine appear to have no direct effect on kinase activity.

2. Purification of myosin light chains

Reverse phase HPLC, which separates molecules on the basis of differing affinities for a hydrophobic resin, has proven quite useful in the purification of low molecular weight peptides, and more recently has found application in the purification of larger proteins. Using a Waters phenyl column and a 0.5% trifluoroacetic acid buffer system with a 43%-49% acetonitrile gradient, we have been able to separate the 17,000 and 20,000 dalton light chains of turkey gizzard myosin from each other, achieving about 100% purity with the latter. We are still in the process of determining the recoverable yield and preservation of the ability of this light chain to be phosphorylated (and remain phosphorylated) following HPLC. The purified protein could be used to develop an antibody for the quantitative determination of light chain phosphorylation in crude tissue extracts. We expect to use this technique to separate the light chains from a variety of tissues (fetal and adult cardiac muscle, platelets, etc.) and species (e.g. Limulus).

3. Biochemical changes associated with inotropy

We are interested in determing whether changes in the contractile state of cardiac muscle myosin are accompanied by changes in phosphorylation of myosin light chains. Rabbits were treated with inotropic agents in doses which significantly raised the ventricular contractility (by Dr. Joanne Urqhart, Cardiology Branch, NHLBI), following which the rabbits' hearts were rapidly frozen. The phosphorylatable light chain will be purified from these hearts using the technique outlined above and the extent of its phosphorylation determined (by glycerol-urea gel electrophoresis).

Publications: Movsesian, M.A.: Calcium physiology in smooth muscle. Progress in Cardiovascular Diseases (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 04202-01 MC

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Studies on the Structure and Function of Myosin Light Chain Kinase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	M. Elizabeth Payne	Staff Fellow	MC NHLBI
Others:	R.S. Adelstein	Chief, Laboratory of Molecular Cardiology	MC NHBLI
	Wm. Anderson, Jr.	Chemist	MC NHLBI
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COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Molecular Cardiology

SECTION

INSTITUTE AND LOCATION
NHLBI - NIH Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.8	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The enzyme myosin light chain kinase contains three domains that interact with one another. These are an active site, responsible for the transfer of phosphate from ATP to myosin; a calmodulin-binding site responsible for the activation of the enzyme by Ca-calmodulin; and 2 sites that can be phosphorylated by cAMP-dependent protein kinase. We are studying the mechanism by which these sites interact with one-another.

723

PHS-6040
(Rev. 2-81)

Project Description:

We are undertaking a study of the topology of the enzyme myosin light chain kinase. This enzyme has at least three domains which can be functionally separated from one another, but which appear to interact with each other in order to regulate enzyme activity. These domains are: a calmodulin binding domain, an active site and two sites (relatively close to each other) which can be phosphorylated by cAMP-dependent protein kinase. Evidence that these domains can be separated from each other comes from a short tryptic digestion of the native enzyme. In the case of gizzard smooth muscle myosin kinase brief tryptic digestion liberates a 22,000 dalton peptide that contains both phosphorylatable sites. The remaining 80,000 dalton protein retains an active catalytic site as well as the calmodulin binding site. Further digestion leads to in the loss of the calmodulin-binding site and results in an enzyme that is completely active in the absence of Ca-calmodulin.

The purpose of the present study is to identify the calmodulin binding site in myosin kinase and to determine how this site interacts with the two phosphorylatable sites. When these latter two sites are occuppied, they weaken the binding of myosin kinase for calmodulin, by about 20-fold.

In order to study these various domains we plan to make use of a number of bifunctional cross-linking reagents as well as monoclonal antibodies, which will be raised to the various sites on myosin kinase. In addition to studying the enzymes isolated from smooth muscle we plan at looking at myosin kinase from non-muscle cells, including normal cells, transformed cells and malignant tumors. By carrying out these structure-function studies we hope to learn how myosin kinase is regulated, and also gain insight into the role of this enzyme in normal and abnormal cells.

Publications:

Adelstein, R.S. and Klee, C.B.: Preparation and purification of smooth muscle myosin light-chain kinase. Methods of Enzymology, 85: 298-308, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 04203-01 MC

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Pathogenesis of hypertrophic cardiomyopathy in man.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I. Michael D. Schneider Medical Staff Fellow MC NHLBI

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INSTITUTE AND LOCATION
NHLBI - NIH Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.45	0.45	0

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To investigate potential mechanisms for the postulated involvement of anti-myocardial cell-surface antibodies in the pathogenesis of human hypertrophic cardiomyopathy (HCM), patients with HCM were assayed by indirect immunoprecipitation for circulating anti-beta-1-receptor autoantibodies. Eight of 18 sera tested resulted in 20-70% precipitation of solubilized turkey erythrocyte beta-1-receptor under the conditions employed. Further work will establish the prevalence of anti-beta-1-receptor autoantibodies in HCM and salient control populations, characterize the antibody-receptor interaction, and assess the ability of anti-beta-1-receptor autoantibodies to function as beta-adrenergic agonists.

PHS-6040
(Rev. 2-81)

We have undertaken to define and resolve cardiac cell surface membrane molecules necessary for development and subsequent regulation of electrical excitability, emphasizing such phenomena as automaticity and receptor-mediated changes in ionic conductances. Methods were investigated to modulate the program of "channel" differentiation. Cardiac cells in tissue culture have been reported to show remarkable plasticity in terms of such critical measures of performance as spontaneous beating and isoenyzme patterns. In particular, properties of the sodium channel and the muscarinic acetylcholine receptor show extraordinary sensitivity to growth conditions, i.e. cells derived from even late, mature embryos revert to primitive, early fetal characteristics.

Cells dissociated from 18-days embryonic chick cardiac ventricles were grown in vitro as monolayers on fibronectin-treated substrata, in the presence of conventional medium containing 10% fetal bovine serum, or in serum-free hormonally-defined medium supplemented with insulin, dexamethasone, and fetuin. As previously reported cardiac myocytes cultured with fetal bovine serum lost the tetrodotoxin sensitivity and rapid sodium influx (150 volts/sec) expected for their age in ovo + in vitro, and concomitantly reacquired spontaneous "pacemaker" activity normally lost after the first embryonic week (Mature myocytes in situ remain electrically excitable but quiescent, and are brought to threshold by specialized conduction tissue.). In contrast, hormonally defined medium suppressed the usual proliferation of cardiac mesenchymal fibroblasts, and the resultant myocytes displayed electrophysiologic properties anticipated for mature cells in situ. Cardiac cells maintained in serum-free medium demonstrate an increase, as fmol/mg protein, in surface membrane receptors for the high-affinity sodium channel ligands saxitoxin and scorpion toxin and in batrachotoxin-stimulated calcium influx. Further work is required (1) to characterize underlying ionic mechanisms, such as available channel number or mean channel open time, responsible for the differential electrophysiologic phenotypes, (2) to investigate the biochemical basis for these responses (Interventions which raise intracellular cyclic AMP levels result in the rapid appearance of new "primitive" sodium channels.).

The molecular basis for cardiac pacemaker activity has not been determined. Even the specific ionic conductance changes which must take place remain controversial; however, cardiac membranes are generally agreed to operate a number of channel species, apart from the classical Hodgkin-Huxley voltage-dependent sodium and potassium channels. One of these, an outward potassium conductance regulated not by transmembrane voltage but rather by intracellular calcium concentration, is regarded as a principal mechanism for membrane oscillation. An 18-amino acid neurotoxin from bee venom, apamin, has recently been reported to exhibit specific inhibition of the calcium-dependent potassium channel in a sympathetic clonal neuroblastoma cell line, and to be a suitable radioligand for high-affinity (10 PM) labelling of neuroblastoma or brain synaptosomal membranes. We have purified apamin by reverse-phase HPLC in TFA/acetonitrile or ammonium acetrate/methanol to obtain a reagent suitable for radioiodination

to study the development of "pacemaker" capacity, affinity, turnover, and receptor-activation coupling in cardiac muscle, and for construction of an apamin-affigel column for affinity chromatography. The specific eluate will be used to generate a library of monoclonal antibodies directed against various channel antigenic domains to elucidate "pacemaker" channel differentiation and function.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 04204-01 MC

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Molecular Events in Cardiac Automaticity and Excitation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	Michael D. Schneider	Medical Staff Fellow	MC NHLBI
Others:	R.S. Adelstein	Chief, Laboratory of Molecular Cardiology	MC NHLBI
	Michael Adler	Senior Staff Fellow	PS NIAAA

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Molecular Cardiology

SECTION

INSTITUTE AND LOCATION
NHLBI - NIH Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.75	0.75	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have undertaken to characterize the cardiac cell surface molecules responsible for regulating ionic conductances in the heart, with particular emphasis on developmental aspects of automaticity ("pacemaker" activity). The reversion to more primitive electrophysiologic behavior (tetrodotoxin-insensitive "slow" sodium channels and spontaneous diastolic depolarization), classically observed with embryonic chick cardiac myocytes grown in monolayer culture in the presence of fetal bovine serum, was prevented in serum-free, hormonally-defined medium that had been supplemented with insulin, dexamethasone, and fetuin. Further work is necessary to establish the biochemical basis and precise ionic mechanisms underlying the succession of electrophysiologic phenotypes during differentiation. A reported high-affinity antagonist of the calcium-dependent potassium channel, the putative "pacemaker", is apamin, a polypeptide neurotoxin from bee venon. Apamin purified by reverse-phase HPLC will be used as a molecular probe for "pacemaker" development, solubilization and affinity chromatography. The specific eluate will be used to produce monoclonal antibodies directed against various channel antigenic domains to elucidate "pacemaker" differentiation and function.

The molecular basis for increased cardiac mass and contractility in hypertrophic cardiomyopathy (HCM) has not been determined. The reported prevalence of anti-myocardial antibodies in HCM, detected by indirect immunofluorescence (Das, Am. J. Cardiol. 25:91, 1970), in contrast to idiopathic cardiomegaly, suggests the participation of immunologic mechanisms at the myocardial cell surface. Agonist-mimetic anti-receptor autoantibodies have recently been identified in a number of human disease states; we previously (Yavin, Yavin, Kohn and Schneider, Cold Spring Harbor Reports in the Neurosciences, 2:141, 1981) generated a library of monoclonal antibodies directed against the glycoprotein component of the thyrotropin receptor, as a means to resolve individual target antigenic domains for Graves' disease autoantibodies and the mechanisms of receptor-activation coupling by which hyperthyroidism occurs. In contrast, neither the specificity of the HCM antibody nor the identity of the antigen recognized was established, and patient sera were not evaluated for salient biological activity against cardiac muscle.

In order to investigate potential mechanisms for the postulated involvement of anti-myocardial cell-surface antibodies in the pathogenesis of human hypertrophic cardiomyopathy, patients with HCM were assayed for circulating antibeta receptor immunoglobulins by indirect immunoprecipitation. Preliminary results, employing solubilized turkey erythrocyte membrane beta-1-receptor (15 fmol/reaction mixture), a 25-fold dilution patient sera, and ^{125}I- iodohydroxybenzopindolol binding for quantitation, indicate that eight of 18 patient sera tested apparently recognize membrane determinants located in or near the ligand binding site of the solubilized beta-1-receptor, under the conditions used yielding 20-70% precipitation. These findings were confirmed with dialyzed patient serum to exclude low molecular weight factors (including drugs such as propranolol) but must additionally be reconfirmed with pure antibody fractions to be obtained by Protein A-Sepharose affinity chromatography.

Further work is required (1) to ensure the molecular identity of the antigen(s) recognized, (2) to characterize the nature and specificity of the interaction between antibody and beta-1-agonist binding, (3) to determine, by antibody-dependent activation of the beta-1-receptor-mediated adenylate cyclase and electrophysiologic study of cultured cardiac myocytes, whether the antibodies function as adrenergic agonists, (4) to define, by peptide mapping and "immunoblotting", a repertoire of beta-1-receptor antigenic structural domains correlating with antibody-mediated physiologic responses, (5) to investigate alternative mechanisms, including direct interaction with cell membrane molecules regulating ionic conductances, whereby anti-myocardial antibodies may influence cardiac performance or structure.

ANNUAL REPORT OF THE
MOLECULAR DISEASE BRANCH
NATIONAL INSTITUTES OF HEALTH
OCTOBER 1, 1981 THROUGH SEPTEMBER 30, 1982

The overall objective of the research program of the Molecular Disease Branch is the delineation of the molecular and structural properties of the human plasma apolipoproteins, the physiological role of the apolipoproteins and lipoproteins in lipid transport, the determination of the mechanisms involved in the regulation of cellular cholesterol metabolism and transport, and the elucidation of the metabolic and molecular mechanisms involved in plasma lipoprotein synthesis, transport, and catabolism in normal individuals and patients with disorders of lipid metabolism and atherosclerosis.

During the last several years the staff of the Molecular Disease Branch has developed a conceptual framework for the understanding of the dynamic processes involved in the biosynthesis, transport, and catabolism of plasma apolipoproteins and lipoproteins. Within this framework the plasma lipoproteins are conceptualized as a polydisperse collection of lipoproteins, the apolipoprotein composition of which is determined by the laws of mass action. The constituent of the lipoprotein particle which is responsible for the regulation of the lipoprotein particle transport and metabolism is the apolipoprotein moiety. The distribution of a specific apolipoprotein within plasma is governed by the relative concentration of and affinity for the individual plasma lipoproteins. This concept of plasma lipoproteins emphasizes the fundamental importance of the apolipoprotein in regulating lipoprotein metabolism and provides a framework for understanding apolipoprotein-lipoprotein interactions during lipoprotein biosynthesis, transport, and catabolism in normal man and in patients with dyslipoproteinemia and atherosclerosis.

Prerequisite to our understanding of the physiological and biochemical role of apolipoproteins in lipid and lipoprotein metabolism is a detailed knowledge of the molecular structure and function of the plasma apolipoproteins. Over the last several years we have undertaken a systematic study of the human plasma apolipoproteins including the primary structure of apoA-I, apoA-II, apoC-I, and apoC-III. Recently, these studies have been extended to an analysis of apolipoproteins $A\text{-}I_{Tangier}$ and $A\text{-}II_{Tangier}$ isolated from patients homozygous for Tangier disease. Detailed studies of the apolipoproteins revealed that $apoA\text{-}I_{Tangier}$ had a change of isoforms with an increase in the $A\text{-}I_2$ and $A\text{-}I_4$ isoforms, increased apparent molecular weight on SDS gel electrophoresis, and a different amino acid composition when compared to apoA-I isolated from normal subjects. ApoA-II isolated from Tangier patients was identical to normal apoA-II. These studies, in combination with metabolic studies on $apoA\text{-}I_{Tangier}$ have established that the molecular defect in Tangier disease is a structural defect in $apoA\text{-}I_{Tangier}$.

Studies on the molecular structure of apoA-I isolated from patients with dyslipoproteinemia have been extended to $apoA\text{-}I_{Milano}$. $ApoA\text{-}I_{Milano}$ was discovered in a kindred of patients in Italy who had mild hypertriglyceridemia and low high density lipoproteins. $ApoA\text{-}I_{Milano}$ contains a cysteine residue which has replaced an arginine residue. This apolipoprotein is therefore structurally different from apoA-I from normal subjects, and is associated with low levels of plasma high density lipoproteins. Kinetic studies on $apoA\text{-}I_{Milano}$ revealed an

increased catabolism when compared to normal apoA-I. This increased catabolism is responsible for the low HDL present in these patients.

During the last year research has continued to focus on apoE. ApoE has been proposed to bind to a high affinity receptor system in the liver, and to be responsible for the uptake of chylomicron remnants by the liver. It is now known that apoE is inherited at a single genetic locus with codominant expression in the population of three common alleles. The alleles (E^2, E^3, and E^4) code for three E apolipoproteins designated $apoE_2$, $apoE_3$, and $apoE_4$. These apolipoproteins have been isolated in homogeneous form and each differ in a single amino acid with a single charge substitution. The in vivo metabolism of these apolipoproteins has been investigated. It now appears that $apoE_3$, the most frequent apolipoprotein in the population, has a different metabolism than the other 2 common E apolipoproteins, E_2 and E_4. $ApoE_2$ is catabolized slower and $apoE_4$ faster in normal man. Studies on the E apolipoprotein in patients with dyslipoproteinemia have revealed a new E apolipoprotein, designated $apoE_{Bethesda}$, which contains two cysteine residues and migrates to the E_1 position on isoelectric focusing. $ApoE_{Bethesda}$ is a new structural variant of the E apolipoprotein.

Of continuing interest over the last year has been a detailed analysis of the B apolipoproteins. Two B apolipoproteins, designated apoB-100 and apoB-48, can be separated on SDS gel electrophoresis. ApoB-100 has been proposed to be sythesized by the liver and is the major protein constituent of LDL. ApoB-48 appears to be synthesized primarily by the intestine, and is a major structural protein of the chylomicron remnant. It has been proposed that apoB-100 and apoB-48 may be utilized as apolipoprotein markers for liver and intestinal lipoproteins respectively. The precise structural relationship between apoB-100 and apoB-48 is not known, however the amino acid composition are not identical. A detailed analysis of the B isoforms in patients with phenotypes I to V was performed. ApoB-48 was present in the very low density lipoproteins in patients with type III and in patients with types I and V if the triglyceride levels were greater than 2000 mg %. These studies, in conjunction with other biochemical and metabolic studies, support the concept that apoB-48 is a marker for intestinal chylomicron remnants. These results will markedly facilitate the categorization of plasma lipoproteins into intestinal or liver lipoproteins.

ApoH is a newly recognized plasma apolipoprotein which is associated with triglyceride-rich lipoproteins in the thoracic duct lymph and plasma. ApoH readily binds to artificial lipid emulsions and has been proposed to have a relatively high affinity for triglyceride-rich lipoproteins. ApoH was shown to activate lipoprotein lipase and to inhibit hepatic lipase. ApoH may therefore play a role in triglyceride hydrolysis in lipoprotein metabolism. It appears therefore that apoC-II, the major cofactor for lipoprotein lipase, and apoH may modulate the activity of enzymes involved in triglyceride metabolism.

The role of apolipoproteins as cofactors for enzymes was further substantiated by studies on the activation of hepatic lipase by plasma. Detailed characterization of the component within plasma responsible for this activation revealed that it was apoA-II. ApoA-II increased the enzymic activity of hepatic lipase two- to three-fold either as an isolated apolipoprotein, recombined with lipids, or as a constituent of HDL. Hepatic lipase is now known to be important in the metabolism of intermediate density lipoproteins and high density lipoproteins. Its major role in lipoprotein metabolism may be as a phospholipase.

ApoA-II may play an important role in lipoprotein metabolism as a cofactor for hepatic lipase catalyzed phospholipid hydrolysis.

The elucidation of the specific physiological and biochemical functions of the apolipoproteins is of pivotal importance in our ultimate understanding of the role of apolipoproteins in lipoprotein structure, function, and metabolism. Currently, apolipoproteins have been shown to be of importance in four general facets of lipoprotein metabolism: 1) cofactor for enzymes (apoC-II and apoH for lipoprotein lipase, apoA-II for hepatic lipase, and apoA-I for lecithin cholesterol acryltransferase); 2) ligand on the lipoprotein particle for interaction with high affinity receptor sites (apoB on LDL and apoE on the chylomicron remnant); 3) exchange protein for phospholipids, cholesterol esters, and triglycerides; 4) structural component for the lipoprotein particle (apoA-I for HDL, and apoB-100 for LDL).

During the last several years significant progress has been made in the development of methods for the solid phase synthesis of large polypeptides and proteins. The use of the phenylacetoamidomethyl (PAM) resin has improved acid stability of coupled proteins, and reduces the rate of peptide loss from the resin during synthesis to only 1% of that of the conventional polystyrene-divinylbenzene resin. The properties of the PAM resin have markedly facilitated the synthesis of large polypeptides and proteins. The PAM resin has been used in our laboratory for the solid phase synthesis of the intact 84 amino acid polypeptide hormone, human parathyroid hormone (h-PTH). A detailed comparison of the synthesized hormone with native h-PTH revealed that the synthetic h-PTH had complete biological as well as immunological activity. The ability to synthesize proteins of 80-100 amino acids in length will permit a detailed analysis of the structure-function requirements of the biological function of several proteins. This will be of particular importance in defining the amino acids in apolipoproteins important in lipid binding, protein-receptor interaction, and enzyme-cofactor function.

A systematic analysis of the molecular properties of the human apolipoproteins continues to be an active research area within the Branch. A detailed knowledge of the molecular properties of the apolipoproteins is required to understand the molecular mechanisms involved in apolipoprotein-lipoprotein particle interaction and metabolism. Within the framework of our concept of lipoproteins the quaternary structure of plasma lipoproteins and the changes in apolipoprotein composition of plasma lipoproteins during transport and metabolism is dependent on the molecular properties of the individual apolipoproteins. Studies performed in our Branch over the last several years have revealed that the human apolipoproteins have unique molecular properties. Of particular interest was the finding that apolipoproteins undergo self- as well as mixed association. Detailed analysis has established the following self-association schemes for the individual apolipoproteins: apoA-I, monomer-dimer-tetramer-octamer; apoA-II; monomer-dimer; reduced and carboxymethylated apoA-II; monomer-dimer; apoC-I, monomer-dimer-tetramer; and apoC-III, monomer-dimer-trimer. Mixed associations between individual apolipoproteins include: apoA-I: apoA-II, 1:1, 2:2; apoA-II: apoC-I, 2:4; and apoA-I: apoC-I, 2:4.

Of particular interest was the discovery during these studies of the marked change in conformation associated with self-association of the apolipoproteins. In the monomeric form, apolipoproteins contain little organized structure whereas in the oligomeric form the conformation increased to that characteristic

of gobular proteins. These dramatic changes observed with self-association are greater than previously reported for any self-associating protein system. The driving force for the dramatic change in conformation of the apolipoproteins is the shielding of non-polar residues from the solvent and is therefore hydrophobic in nature.

The role of mixed association of plasma apolipoproteins may be of major importance in the physiological functions of the individual apolipoproteins. Specific apolipoproteins may be integrated into plasma lipoproteins by protein-protein interactions while other apolipoproteins are associated with lipoprotein particles entirely by protein-lipid interaction. In addition, the mixed protein-protein interactions between plasma apolipoproteins may significantly modulate the physiological function of a given apolipoprotein. The importance of this interaction may be illustrated by the decreased binding of apoE to the apoE-liver receptor in the presence of the C apolipoproteins. The C apolipoproteins appear to modulate the apoE-receptor interaction and may plan an important role in regulating apoE-chylomicron remnant metabolism.

The continued elucidation of the molecular properties of the apolipoproteins and the role of self-association as well as mixed association in apolipoprotein-lipoprotein particle structure, function, and metabolism will be of pivotal importance to our understanding of the quaternary structure of plasma lipoproteins and the function of plasma lipoproteins in normal man and patients with dyslipoproteinemia.

During the last few years the interaction of lipoproteins with cellular receptors has been a major area of research in the lipoprotein field. ApoB-100-LDL interacts with a high affinity receptor on peripheral cells and is responsible for the delivery of cholesterol for cellular metabolism. ApoE-chylomicron remnants interact with a high affinity receptor on the liver resulting in the uptake and catabolism of intestinal particles.

Studies in the Branch have been directed toward an understanding of the nature and specificity of lipoprotein interactions in fibroblasts and liver in normal subjects and patients with dyslipoproteinemia and atherosclerosis. Initial studies were directed toward the analysis of reversible and irreversible lipoprotein-cell interactions. A new microculture technique for growing fibroblasts attached to beads was developed. This assay involved the separation of reversibly-bound lipoproteins associated with cells by rapid filtration. This assay system can now be employed in the analysis of binding affinities and receptor site number involved in lipoprotein-cell interactions.

The interaction of LDL with human liver membranes has been extensively evaluated during the last year. No information was previously available on the role of the liver in the catabolism of LDL in man. These studies have revealed a high affinity, calcium dependent receptor system for LDL on human liver. These results were interpreted as indicating that the liver may play an important role in LDL catabolism in normal man. These studies are consistent with animal studies in which the liver was identified as a major catabolic site for LDL.

The role of the liver in familial hypercholesterolemia has been of great interest for several years. Studies from a number of laboratories have established a receptor defect in peripheral cells (e.g., fibroblasts) in patients with familial hypercholesterolemia. The role the liver receptor in lipoprotein

metabolism in familial hypercholesterolemia, however, was unknown. Recent studies in our Branch have been performed on hepatic LDL binding utilizing liver tissue obtained at surgery in patients with familial hypercholesterolemia. These studies revealed a significant decrease in calcium dependent LDL binding in liver membranes prepared from familial hypercholesterolemic patients. The liver receptor defect therefore parallels the defect previously established in peripheral cells grown in culture. Previous studies from our laboratory and other laboratories have also shown that patients with familial hypercholesterolemia have an increased synthesis of LDL, in addition to their catabolic defect in LDL due to the receptor defect. Based on our results we have proposed that the liver receptor defect is associated with a defective receptor mediated uptake of cholesterol containing lipoproteins which leads to an uptake of lipoproteins by an alternate pathway. The cholesterol contained within LDL taken in by the alternate pathway is not as effective in modulating cholesterol biosynthesis. The increased flux of cholesterol into the liver is associated with an increased synthesis of lipoprotein particles with a hydrated density of LDL. These studies have provided major new insights into the pathophysiology of familial hypercholesterolemia, and may provide additional new information useful in the development of additional modalities of treatment for these patients.

The intracellular transport, hydrolysis, and biosynthesis of cholesterol continues to be an active area of research within the Branch. The interaction and uptake of LDL by the apoB-100 receptor initiates a series of biochemical processes leading to the hydrolysis of cholesterol esters by acid cholesterol ester hydrolase, with the production of free cholesterol. Free cholesterol down regulates HMG-CoA reductase, the rate limiting enzyme in cholesterol biosynthesis, and activates acyl-cholesterol acyltransferase, the enzyme which reesterifies cholesterol to cholesterol ester. Neutral ester hydrolase is the cytosolic enzyme which is responsible for the hydrolysis of cholesterol esters formed by the action of acyl-cholesterol acyltransferase. New sensitive enzymic techniques have been developed in the laboratory for the quantitation of the enzymic activity of acid ester hydrolase, and neutral ester hydrolase. These methods were used in the analysis of these enzymes in fibroblasts grown in culture from a patient with Wolman's disease. Wolman's disease is characterized by steatorrhea, hepatosplenomegaly, absence of acid ester hydrolase and death in early infancy. Analysis of acid ester hydrolase activity in fibroblasts extracts revealed a marked reduction in acid hydrolase enzymic activity, however, the activity of neutral cholesterol ester hydrolase was still present consistent with the concept that the two ester hydrolases were independently coded and synthesized. Studies are currently underway to determine the factors involved in the intracellular regulation of the enzymic activity of both the acid and neutral cholesterol ester hydrolase enzymes.

The regulation of the enzymic activity of HMG-CoA reductase has been extensively studied in our laboratory over the last several years. HMG-CoA reductase has been purified to homogeneity from chicken, rat, and human liver. In all species now studied HMG-CoA reductase was shown to be present in enzymically active and inactive forms. The reversible inactivation of HMG-CoA reductase was shown to be due to covalent modification of the enzyme by a reversible phosphorylation-dephosphorylation reaction sequence. The enzyme, reductase kinase, which catalyzes the phosphorylation of HMG-CoA reductase, has also been purified to homogeneity. Reductase kinase, like HMG-CoA reductase, was shown to undergo reversible activation-inactivation due to reversible

phosphorylation. The kinase responsible for the reversible phosphorylation of reductase kinase has been designated reductase kinase kinase.

Modulation of the degree of phosphorylation and enzymic activity of HMG-CoA reductase has been of particular interest since this mechanism permits the rapid short-term regulation of cholesterol biosynthesis. The polypeptide hormone, glucagon, cholesterol, and mevalonolactone have been shown to modulate HMG-CoA reductase activity by changes in the extent of phosphorylation of HMG-CoA reductase.

Regulation of the enzymic activity of HMG-CoA reductase by a bicyclic cascade system provides a rapid short-term mechanism for the regulation of cholesterol biosynthesis.

An additional mechanism for the regulation of the enzymic activity of HMG-CoA reductase was identified during this last year. Highly purified HMG-CoA reductase was shown to be composed of two isoforms of 52,000 and 54,000 molecular weight. These proteins were isolated and the 54,000 and 52,000 molecular weight species were enzymically inactive and active respectively. The ratio of these two forms differed markedly when analyzed at the nadir of the diurnal rhythm and after cholestyramine feeding. These results suggest that additional mechanisms are involved in the regulation of the enzymic activity of HMG-CoA reductase.

The overall results from these studies clearly establish that the regulation of HMG-CoA reductase is complex and involves a bicyclic cascade system of reversible phosphorylation, covalent modification, and long-term modification involving changes in enzyme synthesis and/or degradation.

The synthesis, transport, and catabolism of plasma lipoproteins in normal subjects and patients with dyslipoproteinemia continues to be actively investigated within the Branch.

Several new diseases have been discovered in patients with dyslipoproteinemia which are due to structural defects or absence of specific plasma apolipoproteins. Two new molecular defects have been associated with type III hyperlipoproteinemia. Previously, studies in our Branch had established that $apoE_2$, coded for by the E^2 allele, was associated with delayed catabolism when compared with $apoE_3$, the most common normal allele for apoE. $ApoE_2$ is the usual E apolipoprotein associated with type III hyperlipoproteinemia, and the majority of the patients with type III hyperlipoproteinemia have been proposed to have a defect in catabolism of chylomicron remnants due to the defective interaction of $apoE_2$ with the hepatic apoE receptor system. The first new defect in type III was the discovery of a patient with a deficiency of the E apolipoprotein associated with a type III phenotype. The proband was a 60 year old female with a 10 year history of xanthomas, 3 year history of angina pectoris, and documented coronary artery disease by angiography. The patient had elevated plasma cholesterol and triglycerides, and a classic type III phenotype on quantification of plasma lipoproteins and lipoprotein electrophoresis. Repeated analysis of plasma and isolated lipoprotein fractions revealed no detectable apoE. ApoE deficiency is therefore a new cause for type III hyperlipoproteinemia.

An additional new molecular defect associated with type III hyperlipoproteinemia was discovered in a 72 year old female. The patient had a long history

of hypertriglyceridemia and hypercholesterolemia, and had characteristic clinical and laboratory findings of a type III phenotype. Analysis of the apoE isoforms by two-dimensional electrophoresis revealed a new apoE variant which migrated in the E_1 position, and was designated $apoE_{Bethesda}$. The patient was heterozygous for $apoE_{Bethesda}$ and the second allele in the patient was E^2. The patient had 1 son whose apoE genotype was $E^{Bethesda}/E^3$. On chemical analysis, $apoE_{Bethesda}$ was shown to contain 2 cysteine residues, similar to $apoE_2$, and therefore appears to be a mutation of the E^2 allele. This new apoE variant demonstrates the marked heterogeneity of the apoE isoforms associated with type III hyperlipoproteinemia.

Of particular interest was a recent study carried out over the last year in which patients with phenotypes I to V were analyzed for the allelic frequency of the E isoproteins. Patients with type III hyperlipoproteinemia had a high frequency of the E^2 allele. In addition, patients with the type V phenotype had an increased frequency of the E^4 allele (57%). The frequencies of the E alleles in phenotypes I, II, and IV were similar to the E allele frequencies ascertained in several normal population studies. In order to determine if the E_4 apolipoprotein in type V patients was functionally normal, metabolic studies were performed in normal individuals and a patient with type V hyperlipoproteinemia. $ApoE_4$ was catabolized at twice the fractional catabolic rate of $apoE_3$. In addition, when compared to $apoE_3$, $apoE_4$ was distributed more in VLDL and less in HDL when analyzed both by column chromatography and ultracentrifugation. These combined results have been interpreted as indicating that $apoE_4$ is metabolically different from $apoE_3$, and may be important in the pathogenesis of type V hyperlipoproteinemia. In addition, these studies would suggest that apoE may have a second metabolic function in addition to facilitating the catabolism of lipoprotein remnants.

During the last year, additional kinetic studies have been performed in patients with type III hyperlipoproteinemia which were homozygous for the E^2 allele. In addition to the reduction of the fractional catabolic rate of $apoE_2$ in type III subjects, the synthesis rate of $apoE_2$ in type III patients was five times greater than the $apoE_3$ synthesis rate in normal subjects. These results indicate that a defect in E metabolism in type III subjects homozygous for the E^2 allele involves both increased synthesis as well as decreased catabolism. The increased plasma E levels in type III patients is due primarily to overproduction with decreased fractional catabolism also playing a significant role.

During the last several years our Branch has extensively analyzed the metabolism of apoA-I and apoA-II, the two major apolipoproteins of HDL. The metabolism of apoA-I and apoA-II was studied by the kinetic analysis of radiolabeled apoA-I and apoA-II. Analysis of simultaneous paired radiolabeled apoA-I and apoA-II studies revealed that the apoA-I residence time was shorter than the apoA-II residence time. In addition, plasma apoA-I levels were correlated with residence time but not synthetic rates, whereas plasma apoA-II concentrations correlated only with residence times. These studies support the concept that apoA-I and apoA-II are not completely linked in metabolism and that at least a fraction of plasma apoA-I and apoA-II are metabolically independent and probably not on the same lipoprotein particle.

Additional studies on apoA-I and apoA-II metabolism revealed that both the residence time of apoA-I and apoA-II were inversely correlated with plasma triglyceride levels. Females also had significantly higher plasma levels and synthesis rates of apoA-I when compared to males. These combined results have

provided important new data on the heterogeneity of the metabolism of apoA-I and apoA-II within HDL, and the factors (e.g., synthesis and catabolic rates) which are important in determining the steady state plasma concentrations of apoA-I and apoA-II.

Research has also continued on the elucidation of the specific molecular defect in patients with dyslipoproteinemia characterized by low plasma HDL. Of particular interest has been the study of Tangier disease, a disease characterized by a striking deficiency of HDL, abnormal triglyceride-rich lipoproteins, and cholesterol ester deposition in the reticuloendothelial system. As noted above, apoA-$I_{Tangier}$ was isolated to homogeneity and differed from normal apoA-I in apparent molecular weight, amino acid composition, and isoform pattern. Additional studies on isolated rat hepatocytes, Kuppfer cells, and endothelial cells indicated that lipoprotein particles containing apoA-$I_{Tangier}$ are taken up much more readily than those containing normal apoA-I. In vivo kinetic studies of radiolabeled apoA-I and apoA-$I_{Tangier}$ demonstrated that the decay of apoA-$I_{Tangier}$ within plasma and HDL was four-fold faster than normal apoA-I when studied in normal volunteers. These combined results were consistent with the view that the molecular defect in Tangier disease is a structural defect in apoA-$I_{Tangier}$ resulting in an abnormally rapid uptake of lipoprotein particles containing apoA-$I_{Tangier}$ by the reticuloendothelial system.

A new and interesting defect in HDL metabolism was discovered in a 45 year old female with corneal opacification, severe coronary artery disease, and normal plasma cholesterol and triglycerides. Analysis of the patient's plasma lipoproteins revealed a striking deficiency of HDL and normal levels of LDL. Apolipoprotein analysis revealed an absence of apoA-I. This new disease entity, apoA-I absence, suggests that a lack of apoA-I is associated with very low levels of HDL and premature cardiovascular disease, supporting the concept that decreased HDL levels are a primary risk factor for the development of atherosclerosis rather than being secondary to increases in plasma triglycerides or chylomicron remnants.

The combined data obtained on the metabolism of radiolabeled apoA-I and apoA-II has been used to develop a compartmental and statistical model for apoA-I and apoA-II metabolism in man. An independent apoA-I model was developed which required the presence of two distinct plasma compartments in both females and males. The major component had a faster residence time (5.4 days) in males than females (6.8 days). The major component which had a faster residence time contained 40% of the apoA-I in females, and 28% in males. ApoA-II had only a single plasma compartment. Analysis of the catabolism of apoA-II revealed that apoA-II decayed by two pathways. One pathway which contained 25% and 30% of the apoA-II in males and females respectively was significantly longer (22 hrs) than the major apoA-II decay pathway. Detailed modeling studies have clearly demonstrated the heterogeneity of the metabolism of apoA-I and apoA-II in both males and females. Detailed analysis of the kinetic studies are critical to our ultimate understanding of the factors modulating the metabolism of apoA-I and apoA-II in normal subjects as well as subjects with dyslipoproteinemia.

Clinical studies are also continuing on triglyceride and fatty acid metabolism in a variety of different populations including diabetic and non-diabetic Pima indians and patients with familial hypertriglyceridemia and combined hyperlipidemia. In diabetic Pima indians the synthesis rate of VLDL-triglycerides did not correlate with plasma free fatty acid levels or with

changes in free fatty acid levels. The VLDL-triglyceride production rate was strongly positively correlated, however, with plasma C-peptide levels suggesting that insulin may play a major role in short-term modulation of triglyceride synthesis in diabetes.

Studies have also been continued during the last year on the interesting disease, betasitosterolemia, which is characterized clinically by xanthomas in early childhood, premature cardiovascular disease, and elevated plasma sterol levels. Recent metabolic balance studies have shown that these patients have an increased absorption of shellfish sterols, as well as the previously demonstrated overabsorption of cholesterol and plant sterols. These combined data now indicate that these patients have a pansterol metabolic abnormality leading to increased absorption of all sterols and deposition of these sterols in tissue leading to premature cardiovascular disease.

One of the major aims of the staff of the Branch is the effective treatment of hyperlipidemia with the ultimate goal of reducing blood levels at an early stage of atherosclerosis and preventing premature cardiovascular disease. To this end we have initiatated an ongoing outpatient clinical trial for the treatment of patients with hypercholesterolemia and the type II phenotype with neomycin as the hypocholesterolemic drug. Previous drug regimes including cholestyramine and nicotinic acid have been poorly tolerated by patients due to side effects of the medications. Preliminary results from the neomycin study are encouraging and suggest that this drug is well tolerated and therapeutically useful.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02003-11 MDB

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The Biochemistry and Metabolism of Plasma Lipoproteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH
Molecular Disease Branch

SECTION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.8	1.8	2.0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Research has focused on plasma apolipoprotein (apo) composition and metabolism in normal and dyslipoproteinemic subjects. Patients with Tangier disease have an abnormal apoA-I (apoA-ITangier), which differs from normal apoA-I in amino acid composition, molecular weight, and in vitro and in vivo metabolism. ApoA-I absence, an autosomal recessive disorder, is associated with undetectable plasma concentrations of apoA-I. The interaction of apoA-I and apoA-II with plasma lipases is also being investigated. ApoA-II has been shown to activate hepatic lipase enzymic activity. Ongoing studies of apoE metabolism support the concept that most patients with type III hyperlipoproteinemia (HLP) have an abnormal apoE (E2). In addition, patients with type III HLP associated with a deficiency of apoE have recently been found, and represent a new cause for this form of HLP. In addition we have noted a much higher prevalence of apoE4 homozygosity in type V hyperlipoproteinemic patients, as compared to normals. Various dyslipidemic states have therefore been associated with specific apolipoprotein abnormalities.

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Project Description:

Objectives:

1) To complete studies on apoA-I, apoA-II and apoE metabolism in normal and dyslipoproteinemic man.

2) To screen patients with various forms of dyslipoproteinemia for an absence or structural abnormality of apolipoproteins.

3) To determine the effects of various diets and drugs on plasma lipoproteins and apolipoproteins in normal and dyslipoproteinemic subjects and to study the clinical features of these latter subjects.

4) To study the interaction of apolipoproteins, various enzymes (lipoprotein lipase, hepatic triglyceride lipase) and plasma lipoproteins.

Methods Employed:

Methodology for the isolation, quantitation, and radioiodination of plasma lipoproteins and apolipoproteins has been previously described. Methodology for plasma apolipoprotein determinations have been developed by other members of our branch. We have implemented a computerized record keeping system for purposes of storing and analyzing clinical and lipoprotein data on our patients.

Major Findings:

1) The kinetics of the major apolipoproteins (apo) of plasma high density lipoproteins (HDL), apoA-I and apoA-II, were examined in a total of 44 individual tracer studies in 22 normal male and female subjects (1). Following the intravenous injection of radioiodinated HDL, the specific radioactivity decay of apoA-I within HDL (residence time, 5.07 $\pm$ 1.53 days), as determined by column chromatography, was significantly ($P < 0.01$) faster than that of apoA-II (residence time, 5.96 $\pm$ 1.84 days). The specific radioactivity decay of apoA-I within HDL when labeled on HDL or as apoA-I was found to be almost identical. Similar results were obtained for apoA-II. Analysis of simultaneous paired radiolabeled apoA-I and apoA-II studies revealed that the mean apoA-I plasma residence time (4.46 $\pm$ 1.04 days) was significantly ($P < 0.01$) shorter than that for apoA-II (4.97 $\pm$ 1.06 days). Females had significantly ($P < 0.01$) higher apoA-I plasma concentrations (124 $\pm$ 24 mg/dl) and apoA-I synthesis rates (13.58 $\pm$ 2.23 mg/kg·day) than did males (108 $\pm$ 16 mg/dl, and 11.12 $\pm$ 1.92 mg/kg·day, respectively). Plasma apoA-I levels were correlated with plasma apoA-I residence times, but not synthesis rates; and apoA-II concentrations were correlated only with apoA-II whole body residence times. ApoA-I and apoA-II plasma residence times were inversely correlated with plasma triglyceride levels. These data are consistent with the following concepts: 1) labeling of apoA-I and apoA-II as apolipoproteins or on HDL does not affect their specific radioactivity decay within HDL; 2) the mean residence time of apoA-I both in plasma and in HDL is significantly shorter than that of apoA-II; 3) the increased apoA-I levels seen in female subjects are due to increased apoA-I synthesis; and 4) the plasma apoA-I residence time, which in inversely correlated with plasma triglyceride levels, is an important determinant of apoA-I concentration in both males and females.

2) Tangier disease, originally described by Fredrickson and associates, is a rare autosomal recessive disorder characterized by a markedly decreased HDL, abnormal triglyceride-rich lipoproteins, and cholesterol ester deposition in reticuloendothelial cells throughout the body, especially in the tonsils, lymph nodes and spleen. Tangier homozygotes have apoA-I and apoA-II levels which are approximately 1% and 5% or normal, respectively. Other apolipoprotein levels are normal or only slightly reduced in these patients (2). We have previously reported that Tangier heterozygotes and homozygotes have a striking hypermetabolism of apoA-I and apoA-II which accounts for the reduced levels seen in the plasma of these patients.

Ongoing studies in our branch are being carried out in an effort to determine the precise nature of the defect in Tangier disease. Recently we have noted that apoA-I isolated from Tangier homozygotes differs from normal apoA-I in its molecular weight, amino acid composition, and isoform pattern (see annual report of Dr. H. Bryan Brewer, Jr.) (3). In addition, in vitro studies utilizing isolated rat hepatocytes, Kuppfer cells, and endothelial cells, indicate that lipoprotein particles containing apoA-$I_{Tangier}$ are taken up much more readily than those containing normal apoA-I. In vitro lipoprotein binding studies indicate that apoA-$I_{Tangier}$ does bind to normal lipoprotein particles, but has somewhat different binding characteristics than does normal apoA-I (4). In vivo metabolic studies have demonstrated that the specific radioactivity decay within plasma and HDL in four normal subjects was approximately three fold enhanced for apoA-$I_{Tangier}$ as compared to normal apoA-I (4). These data are consistent with the concept that patients with homozygous Tangier disease have an abnormal apoA-I (apoA-$I_{Tangier}$) which results in abnormal lipoproteins with uptake by reticuloendothelial cells, and consequent cholesterol ester deposition, as well as striking hypercatabolism of apoA-I, and HDL deficiency (4).

3) A 45 year old woman with corneal opacification and severe coronary artery disease was noted to have the following plasma lipid levels (mg/dl $\pm$ SD): total cholesterol 111 $\pm$ 13, triglyceride 62 $\pm$ 6, very low density lipoprotein cholesterol 4 $\pm$ 1, low density lipoprotein cholesterol 106 $\pm$ 14, and high density lipoprotein (HDL) cholesterol 1 $\pm$ 1 (normal, 50 $\pm$ 14) (5). Her two offspring and one brother were found to have HDL cholesterol values (mg/dl) of 23, 20, and 20, respectively. The percentage of cholesterol in the esterified form in the patient's plasma was normal at 70%. Lipoprotein electrophoresis showed no alpha lipoprotein band, and no HDL was detectable when plasma was subjected to analytic ultracentrifugation. Only trace amounts of lipids were noted within the HDL density region following preparative ultracentrifugation. Mean plasma apolipoprotein (apo) A-II, apoB, and apoC-II plasma levels were 13.8%, 130.6%, and 26.6% of normal, respectively. The ratio of apoB to cholesterol within LDL was elevated. ApoA-I, the major HDL protein constituent, was immunologically undetectable in this patient's plasma. A decreased HDL cholesterol concentration has been associated with premature coronary artery disease. These data indicate that plasma apoA-I absence results in a striking reduction in HDL, is associated with premature coronary artery disease, and represents a new distinct disease entity. The data suggest that apoA-I is not essential for cholesterol esterification. In addition the clinical features in this disease support the concept that decreased HDL levels are a primary risk factor for atherosclerosis rather than being secondary to increases in plasma triglycerides or chylomicron remnants.

4) The effect of various apolipoproteins on the enzymic activity of lipoprotein lipase (LPL) and hepatic triglyceride lipase (HTGL) has been studied. ApoC-II, a protein constituent of human very low density lipoproteins (VLDL) and HDL, has previously been shown in our branch to activate LPL activity. LPL is necessary for triglyceride hydrolysis in plasma. ApoH, a protein constituent of humna lymph chylomicrons, found in plasma in the lipoprotein free fraction (density > 1.21 g/ml) and also known as β_2-glycoprotein-1, has been shown in our branch to augment LPL activity 2-3 fold in the presence of apoC-II. The function(s) of HTGL are as yet not fully elucidated. This enzyme appears to hydrolyze both triglyceride and phospholipids, and HTGL's most important role may be as a phospholipase. In this respect, HTGL may have a significant function in the breakdown of phospholipids on VLDL and HDL. Recently we have shown that apoA-II, a major HDL protein constituent, activates HTGL enzymic activity (see Annual Report of Dr. James C. Osborne) (6). This finding may be of particular importance in understanding HDL metabolism, since HL may be important in the conversion of HDL_2 to HDL_3.

5) Subjects with type III hyperlipoproteinemia develop premature atherosclerosis and have hyperlipidemia due to an increase in cholesterol-rich very low density lipoproteins (VLDL) of abnormal electrophoretic mobility. Apolipoprotein E is a major protein constituent of VLDL and appears to be important for the hepatic uptake of triglyceride-rich lipoproteins. A new kindred of patients with type III hyperlipoproteinemia is described in which no plasma apolipoprotein E could be detected, consistent with the concept that type III hyperlipoproteinemia may be due to an absence or striking deficiency of apolipoprotein E (7). Members of the kindred with type III HLP described, because of their lack of detectable plasma apoE, differ from other known type III HLP kindreds, all of which have shown increased amounts of an abnormal apoE. In addition, these subjects had only mild hypertriglyceridemia, increased LDL cholesterol, and a much higher ratio of VLDL cholesterol to plasma triglyceride than reported in other type III HLP subjects. Apolipoprotein A-IV and lower molecular weight apoB (B-48), two lymph chylomicron apolipoprotein constituents generally not found in normal plasma IDL and LDL, were present in significant quantities in the IDL and LDL of these patients with apoE deficiency. These data are consistent with the following concepts: (i) apoE is important for the catabolism of chylomicron remnants; (ii) apoE deficiency results in the accumulation of chylomicron remnants in plasma, type III HLP, tubo-eruptive xanthomas, and premature coronary artery disease; and (iii) apoE deficiency represents a new disease entity.

6) The therapy of patients with type III hyperlipoproteinemia has also been studied (8). Type III patients with either $apoE_2$ homozygozity or apoE deficiency experience a significant decrease in their plasma lipoprotein levels with low cholesterol, low saturated fat diets with restriction of simple carbohydrates. Most patients require drug therapy with either nicotinic acid 1 gram po tid or clofibrate 1 gram p.o. b.i.d. to normalize their plasma lipids on an outpatient basis.

7) Type V hyperlipoproteinemia (HLP) is characterized clinically by hepatosplenomegaly, occasional eruptive xanthomas, and an increased incidence of pancreatitis. These patients have striking hypertriglyceridemia due to increased plasma chylomicron and very low density lipoprotein concentrations in the fasting state, without a deficiency of lipoprotein lipase or its activator protein, apo-

lipoprotein (apo) C-II. ApoE, a protein constituent of triglyceride-rich lipoproteins, has been implicated in the receptor-mediated hepatic uptake of these particles. ApoE has three major alleles E^2, E^3, and E^4, and the products of these alleles are $apoE_2$, $apoE_3$, and $apoE_4$, respectively. ApoE phenotypes were determined in 30 type V HLP patients as well as in 37 normal volunteers (9). Among the type V patients, 33.3% were noted to be homozygous, and 40.0% heterozygous for E^4 (normal, 2.7 and 21.6%, respectively). These data suggest that $apoE_4$ may play a role in the etiology of the hyperlipidemia in a significant number of type V HLP patients.

8) The apolipoprotein E (apoE) phenotype has been studied by slab gel isoelectrofocusing of delipidated very low density lipoproteins (VLDL, $d < 1.006$ g/ml) in a group of normal individuals (n=74) and a group of patients with hyperlipoproteinemia (n = 226) (10). ApoE has been proposed to be inherited at a single genetic locus with three common alleles designated E^2, E^3 and E^4. The three gene apoprotein products are $apoE_2$, $apoE_3$ and $apoE_4$, and three homozygous (E_2, E_3 and E_4) and three heterozygous ($E_{2/4}$, $E_{2/3}$ and $E_{3/4}$) apoE phenotypes may be ascertained by gel isoelectrofocusing. In the present study, all the apoE patterns could be assigned to one of the six apoE phenotypes. In the normal population the following phenotypic distribution was observed: E_3: 55.4%; $E_{3/4}$: 21.6%; $E_{2/3}$: 18.9%; and E_4: 4.1%. No subject expressing the E^2 or the E^2/E^4 genotypes were found. Patients with Type I, Type IIa, Type IIb and Type IV hyperlipoproteinemia had an apoE phenotypic distribution which was similar to normal. In contrast, the apoE phenotype was significantly different in patients with type III and type V hyperlipoproteinemia. In type III subjects, nine of 12 (75%) had an E_2 phenotype and three of 12 were E^2 heterozygotes. Eleven of 35 (31.4%) patients with Type V hyperlipoproteinemia had an E_4 phenotype, and an additional 15 (43%) were E^4 heterozygotes. No E_3 phenotype, the most commonly encountered phenotype among normals and the other groups of hyperlipoproteinemic subjects was observed in Type III and a very low frequency (5.7%) was seen among Type V patients. The combined results are interpreted as indicating that two major apoE isoproteins, $apoE_2$ and $apoE_4$, are associated with two distinctly different dyslipoproteinemias, possibly indicating at least two different physiological functions for apoE. These data are consistent with the concept that $apoE_2$ and $apoE_4$ are common abnormal mutants of $apoE_3$ which predispose to dyslipidemia.

9) The effects of various cholesterol-lowering diets on plasma lipid and lipoprotein cholesterol levels were assessed in normal and hypercholesterolemic subjects. The base-line diet was an ad libitum hospital diet of normal composition (11). Diet A was a 20% protein, 40% carbohydrate, 40% fat, polyunsaturated: saturated fat ratio 0.1 to 0.3, 250 to 300 mg cholesterol diet, diet B was identical to diet A except that the polyunsaturated/saturated fat ratio was 1.8 to 2.2, and diet C was a 20% protein, 80% carbohydrate, very low fat (5 to 10 g), polyunsaturated/saturated fat ratio 0.1 to 0.3, 150 to 200 mg cholesterol diet. Diet A (low cholesterol) caused mean reductions in plasma, low density lipoprotein (LDL), and high density lipoprotein (HDL) cholesterol of 5.9, 5.6, and 6.3%, respectively, in 11 normal subjects. Diet B (low cholesterol, high polyunsaturated fat) caused significant decreases in plasma cholesterol, LDL cholesterol and HDL cholesterol of 17.0, 16.2, and 17.4%, respectively, in 12 normal subjects; and reductions of 11.0, 10.8, and 17.1%, respectively, in 19 hypercholesterolemic subjects. Diet C (low cholesterol, very low fat) produced significant mean decreases in plasma, LDL, and HDL cholesterol of 26.7, 29.9, and 27.9%, respec-

tively, in 11 normal subjects, and in nine hypercholesterolemic patients of 22.6, 27.2, and 28.6%, respectively. The reductions in plasma cholesterol observed with these diets were therefore due to decreases in both LDL and HDL cholesterol with no significant changes in the LDL cholesterol:HDL cholesterol ratio.

10) The metabolism of apolipoproteins in relationship to high density lipoprotein metabolism (12) and lipoprotein abnormalities (13) has recently been reviewed.

11) It has been reported that hypercholesterolemic patients have enhanced platelet aggregability (especially in response to epinephrine) and decreased platelet survival, and it was suggested that these factors might play a role in the pathogenesis of the premature atherosclerosis seen in these patients. Over the past several years, in collaboration with Dr. Lawrence Corash, Hematology Department, Clinical Center, we studied platelet aggregation in heterozygous and homozygous familial hypercholesterolemic patients, as well as in normal subjects. Heterozygotes were older, had more diffuse and advanced atherosclerosis, and lower plasma cholesterol concentrations than did homozygotes. Homozygotes that were studied had only limited atherosclerosis. In these studies normals and homozygotes in general had normal platelet aggregation and survival, while heterozygotes have enhanced aggregation and shortened survival. These data are consistent with the view that platelet abnormalities seen in familial hypercholesterolemia are not related to plasma cholesterol concentrations but to the degree of atherosclerosis present (14).

12) Plasma lipids and lipoproteins were studied at presentation in 25 patients with acute leukemia and non-Hodgkin's lymphoma (15). All patients demonstrated an abnormality in at least one plasma lipid fraction, and most exhibited a predictable pattern of lipid alterations that consisted of extremely low levels of high density lipoprotein cholesterol (median [Xm] = 23), elevated triglyceride (Xm = 165) and elevated very low density lipoprotein (Xm = 26). Patients restudied during remission demonstrated a return to normal values. The degree of lipid abnormality was directly related to the underlying tumor burden and particularly to the presence of bone marrow involvement. However, even patients with minimal tumor burden demonstrated plasma lipid abnormalities. The results suggest that an abnormality in systemic lipid metabolism, possibly in triglyceride clearance, is present in these patients and that its incidence in this population is high.

13) In collaboration with Dr. Dennis Sprecher, patients with premature coronary artery disease are being screened for lipoprotein and apolipoprotein abnormalities by standard lipoprotein determination as well as two dimensional gel electrophoresis utilizing silver staining. In addition the clinical, biochemical, and necropsy features of NIH patients with homozygous familial hypercholesterolemia (FH) were reviewed.

14) In collaboration with Dr. Jeffrey Hoeg the hepatic membrane binding or radioiodinate LDL in normal and FH livers is being assessed. Preliminary data suggest that these patients have a hepatic LDL receptor defect as well as a defect in their fibroblast receptor.

Significance to Biomedical Research and the Program of the Institute:

Lipoproteins have been implicated in the pathogenesis of atherosclerosis. One of the missions of the intramural research program of the National Heart, Lung, and Blood Institute is to elucidate the mechanisms and factors which lead to atherosclerosis, the leading cause of death in the U.S. society. The current leading concept as to how atherosclerosis arises is that factors such as hypertension and smoking predispose to arterial endothelial cell damage, that low density lipoproteins and chylomicron remnants are deposited at such sites of damage, while HDL is a protective factor which decreases the amount of cholesterol deposition in the arterial wall. Over the years this process leads to significant lipid deposition, smooth muscle cell proliferation, calcification, narrowing, and ultimately blockage of the major arteries of the body. Since most patients with coronary heart disease or cerebro-vascular disease die suddenly outside the hospital, and since surgical procedures such as bypass grafting do not have a significant effect on longevity because atherosclerosis is generally a diffuse process, individuals at increased risk for atherosclerosis should be diagnosed relatively early in life. Researchers in the Molecular Disease Branch are in a unique position to elucidate molecular mechanisms for dyslipidemia, which predispose patients to premature atherosclerosis.

Members of our branch have had significant success in elucidating such mechanisms over the past several years. Increased levels of HDL, as observed in females, are associated with a decreased risk for atherosclerosis as well as increased plasma levels of apoA-I and apoA-II. Our studies indicate that these increased levels observed in females are due to increased synthesis of apoA-I and apoA-II, and that this enhancement is mediated by estrogens (1). In addition hypertriglyceridemia and obesity lead to decreased HDL levels by causing an increased rate of clearance of HDL constituents. HDL levels are modulated in part by hepatic lipase which is estrogen and androgen sensitive. Recently we have shown that this enzyme is activated by apoA-II (6).

Tangier disease, described over 20 years ago by Fredrickson and colleagues, is associated with cholesterol ester deposition, premature atherosclerosis, and decreased HDL, apoA-I, and apoA-II plasma concentrations (2). We reported several years ago that patients with this classic HDL deficiency disease, had relatively normal synthesis of apoA-I and apoA-II, but rapid and disparate catabolism of these apolipoproteins, with apoA-I being cleared from the plasma approximately four fold faster than apoA-II. Recently we have isolated Tangier apoA-I and shown that it has a different molecular weight and amino acid composition (3) as well as significant enhanced in vivo catabolism (4) as compared to normal apoA-I. Thus Tangier disease whose biochemical defect so long remained elusive, now has been shown to be due to a structural abnormality of apoA-I ($apoA\text{-}I_{Tangier}$).

Another disease entity was recently described by members of our branch. This autosomal recessive disorder named apolipoprotein A-I absence is associated with severe premature CAD, mild corneal opacification, marked HDL deficiency, and undetectable plasma apoA-I (5). This disease further confirms of decreased HDL as a significant independent primary risk factor for atherosclerosis.

The finding that apoA-II, the other major protein of HDL, activates hepatic lipase (6) may have important implications for furthering our understanding of the metabolism of both triglyceride rich lipoproteins as well as HDL.

Another apolipoprotein, apoE, a protein constituent of triglyceride rich lipoproteins, has been implicated in the receptor mediated hepatic uptake of these lipoproteins. Previous studies from other laboratories have shown that patients with type III hyperlipoproteinemia (increased intermediate density lipoproteins) are likely to have an abnormal apoE phenotype (apoE2) while most normal subjects have the apoE3 phenotype.

Recently we have described a kindred with premature coronary artery disease and type III hyperlipoproteinemia associated with apoE deficiency, confirming the importance of apoE in the hepatic uptake of chylomicron remnants (7). Moreover our experience with therapy of type III hyperlipoproteinemia has recently been summarized (8), indicating that both diet and drug therapy are crucial in this disease.

Type V hyperlipoproteinemia (increased chylomicrons and very low density lipoproteins), with significant hypertriglyceridemia, is another dyslipidemic state, that we have recently associated with an apoE abnormality. A significant percentage of type V patients (approximately 70%) are either homozygous or heterozygous for apoE4, a form of apoE rarely observed in normals (9,10). Thus apoE abnormalities have now been associated with two forms of hyperlipoproteinemia (type III and type V).

Diet is the cornerstone of therapy for hyperlipidemia. The effects of low cholesterol, high polyunsaturated fat, and low fat diets on plasma lipoproteins have been assessed in normal and hypercholesterolemic subjects (11). These studies clearly indicate that cholesterol restruction, an increase in the polyunsaturated fat ratio, and fat restriction are all important dietary alterations in defining the ideal lipid lowering diet.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02010-11 MDB

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Structure and Function of Plasma Lipoproteins and Apolipoproteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH
Molecular Disease Branch

SECTION
Peptide Chemistry

INSTITUTE AND LOCATION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.3	2.3	3.0

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Apolipoprotein A-I has been isolated, and characterized from patients homozygous for Tangier Disease. ApoA-Tangier were shown to have increased isoform heterogeneity, larger apparent molecular weight, and different amino acid composition when compared to normal apoA-I. These studies in conjunction with metabolic studies indicate that the molecular defect in Tangier disease is a structural defect in apoA-I-Tangier. The polymorphic forms of apoB (B-48, B-100) have been characterized in patients with phenotypes I to V. B-48 was present in VLDL patients with phenotypes I and V when triglycerides were greater then 1000, and in all type III patients. B-48 appears to be a metabolic marker for intestinal lipoproteins.

Human parathyroid hormone (1-84) has been synthesized by solid phase techniques. The synthetic hormone is identical to the native hormone in physico-chemical properties and biological activity.

748

Project Description:

Objective:

1) Characterization of the polymorphic forms of apoB in plasma and thoracic duct lymph.

Methods Employed:

Plasma and lymph apoB can be separated into two polymorphic forms of different apparent molecular weight by sodium dodecyl sulfate electrophoresis. These two forms have been designated as B-100 and B-48 and represent the higher and lower apparent molecular weight forms of apoB respectively. B-100 can be purified from LDL, and B-48 can be isolated by preparative SDS gel electrophoresis from delipidated human thoracic duct lymph.

Major Findings:

B-100 and B-48 have been isolated in homogeneous form from delipidated LDL and thoracic duct lymph respectfully. The apparent molecular weight of B-100 and B-48 are approximately 300,000 and 180,000. The amino acid composition of B-100 and B-48 reveal distinct differences in composition, and suggest that the two apolipoproteins are not different oligomeric forms of the same protein subunits. Antibodies prepared against B-100 and B-48 have shown cross reactivity with some antisera, and unique specificity with other antisera. These results have been interpreted as indicating that B-48 and B-100 may have similar antigenic sites, however the presence of antisera which show no cross-reactivity indicate that the covalent structure of the two forms of apoB are not identical. Detailed chemical and physical characterization is currently underway.

2) Determination of the content of B-100 and B-48 in plasma lipoproteins of patients with dyslipoproteinemia.

Methods Employed:

Patients with hyperlipoproteinemia (N=60) attending the outpatient clinic were selected to participate in the study. The lipoproteins from 10 patients with phenotypes I, IIA, IIB, III, IV, and V were analzyed. The plasma lipoproteins were separated into chylomicron - VLDL, IDL, LDL, and HDL by sequential ultracentrifugation. The presence of B-100 and B-48 was determined by 3.5% SDS polyacrylamide gel electrophoresis.

Major Findings:

Patients with type I and type V hyperlipoproteinemia could be separated into two groups by the degree of hypertriglyceridemia and the presence or absence of B-48. Patients with triglycerides <1000 mg/dl with both type I and type V hyperlipoproteinemia contained only B-100 in all lipoprotein density classes. In the second group of patients in both phenotypes with triglycerides greater than 2000 mg/dl B-48 and B-100 was present in the chylomicron-VLDL density class, however B-100 was the only lipoprotein in IDL and LDL. In type III patients B-48 and B-100 were present in the chylomicron-VLDL fraction, with only B-100 in

IDL and LDL. One patient with type III hyperlipoproteinemia due to apoE absence was unique and had B-48 and B-100 in chylomicron VLDL, IDL, and LDL. Patients with types IIA, IIB, and IV had only B-100 in all density fractions. Patients (n=3) with homozygous familial hypercholesterolemia were of particular interest due to their high plasma concentration of LDL. These patients have elevated LDL levels due to increased LDL synthesis, and defective catabolism due to a defect in the LDL receptor. B-100 was the only B apolipoprotein in all density fractions in patients homozygous for familial hypercholesterolemia. The combined results from these studies can be interpreted as indicating that B-48 can be utilized as a metabolic marker for lipoproteins of intestinal origin. The high triglyceride and chylomicron levels in patients with type I and V hyperlipoproteinemia are due to delayed clearance of chylomicrons. In type III patients B-48 also indicates delayed clearance of chylomicron remnants. The lack of B-48 in the IDL-LDL fraction of patients with homozygous familial hypercholesterolemia suggests that the increased biosynthesis of these particles is of hepatic origin.

3) Isolation and purification of apoC-II from normal subjects.

Methods Employed:

ApoC-II is the major apolipoprotein cofactor for lipoprotein lipase, the major enzyme involved in hydrolysis of lipoprotein triglycerides. ApoC-II has not been isolated and characterized from normal subjects. Plasma of density <1.006 g/ml from normal subjects was pooled and the apoC-II fraction isolated by DEAD chromatography, followed by high pressure liquid chromatography using reverse phase.

Major Findings:

ApoC-II isolated by conventional DEAE chromatography was not homogeneous by basic polyacrylamide gel electrophoresis in 8 M urea. Final purification involved the development of a new chromatographic procedure utilizing high pressure liquid chromatography (HPLC) with reverse phase. The HPLC purified apoC-II was homogeneous by SDS gel electrophoresis, and basic polyacrylamide gel electrophoresis in 8 M urea. The amino acid composition of apoC-II from normal subjects is not identical to that from patients with dyslipoproteinemia suggesting that there may be structural heterogeneity of apoC-II which may be of functional significance.

4) Isolation and characterization of apoA-II and apoA-II from patients homozygous for Tangier Disease.

Methods Employed:

Studies have continued on the isolation, characterization and functional analysis of apoA-I and apoA-II isolated from patients who are homozygous for Tangier disease. The methods involved in the isolation and characterization of apoA-$I_{Tangier}$ and apoA-$II_{Tangier}$ are detailed in last year's annual report and involved isolation of lipoproteins by ultracentrifugation, and purification of apolipoproteins by preparative SDS gel electrophoresis and/or DEAE chromatography.

Major Findings:

ApoA-$I_{Tangier}$ isolated from Tangier patients by gel permeation chromatography and preparative SDS gel electrophoresis was compared to apoA-I isolated from HDL by similar techniques. ApoA-$I_{Tangier}$ had greater heterogeneity of isoforms by isoelectric focusing and a slightly greater apparent molecular weight by SDS gel electrophoresis when compared to normal apoA-I. In addition the amino acid composition of apoA-$I_{Tangier}$ had several amino acids which were different from the amino acid composition of normal apoA-I. ApoA-II from Tangier patients isolated by gel filtration followed by ion exchange chromatography was identical to normal apoA-II in chemical and electrophoretic properties, and had an amino acid composition similar to that of normal apoA-II. These studies have now been extended to the metabolism of radiolabeled apoA-$I_{Tangier}$ and apoA-$II_{Tangier}$ in normal subjects and patients with Tangier disease. In these studies apoA-$I_{Tangier}$ had a very rapid catabolism when compared to normal apoA-I, however the metabolism of apoA-II from normal subjects and Tangier patients was identical. The combined results from these studies have been interpreted as indicating that the molecular defect in Tangier disease is due to a structural defect in apoA-$I_{Tangier}$.

5) Solid phase synthesis of human parathyroid hormone.

Methods Employed:

Human parathyroid hormones (h-PTH) of an 84 amino acid polypeptide has been synthesized by the Merrifield solid phase technique using the phenylacetaminomethyl (PAM) resin. The details of the initial synthesis of this polypeptide were included in last year's annual report. Briefly the synthesis involved the use of the PAM resin which reduces the rate of peptide loss from the resin by acidolysis to only 1% of that of the conventional styrene-divenylbenzene resins and permits the synthesis of larger proteins than conventional methodology. During the synthesis t-BOC groups were used to protect the α-amino groups of all amino acids except arginine where the more soluble amyloxy derivative was employed. Initially a 25% solution of TRA in CH_2Cl_2 was used for the protection of the 2-amino groups; at step 40 the concentration of TRA was increased to 40%. Amino acids were detached to the peptide-resin by coupling with dicyclododecyl carbodimide (DCC) utilizing t-BOC amino acids. The active ester method was used to couple the p-nitrophenyl esters of asparagine and glutamine. The completed protected peptide was cleaved from the resin by anhydrous hydrogen fluoride. The synthetic protein was purified by gel filtration followed by ion exchange chromatography.

Major Findings:

A detailed study of the chemical, immunological, and biological properties of synthetic h-PTH has now been carried out. Synthetic h-PTH was a single band when analyzed by SDS gel electrophoresis and acid polyacrylamide gel electrophoresis (pH 4.4) in 8 M urea. The electrophoretic properties of synthetic h-PTH were identical to native h-PTH. The amino acid composition of synthetic h-PTH and native h-PTH were virtually identical. Amino terminal sequence analysis of synthetic h-PTH revealed a single unique sequence with no major deletions or overlaps. The biological activity was analyzed by the activation of adenyl cyclose in rat kidney membranes, and a purified receptor membrane system prepared from dog kidney tissues. Synthetic h-PTH had essentially the same biological

activity of native h-PTH indicating that the hormone had been synthesized with virtually complete biological activity. The immunological activity of synthetic h-PTH was determined utilizing antisera specific for the carboxyl terminal region of the hormone as well as antisera which had determinants to the intact protein. Synthetic h-PTH had a high degree of immunoreactivity for both assay systems when compared to native h-PTH. These combined studies indicate that the synthetic h-PTH had chemical, biological, and immunochemical properties which were very similar to those of the native h-PTH hormone.

Significance to Biomedical Research and the Program of the Institute:

The isolation, characterization, and sequence analysis of the human plasma apolipoproteins is an ongoing program designed to ultimately understand the physiological role and molecular mechanisms involved in the synthesis, transport, and metabolism of plasma lipoproteins in normal individuals and patients with disorders of lipid transport and atherosclerosis.

Proposed Course of the Project:

The isolation, characterization and structural analysis of plasma apolipoproteins and apolipoproteins within human thoracic duct lymph will be continued. Major emphasis will be continued on apolipoproteins E, H, A-IV, B-48, and B-100. The defect in the covalent structure of apoA-$I_{Tangier}$ will be pursued with the ultimate goal of determining the amino acid sequence of apoA-$I_{Tangier}$. The isolation and sequence of apoC-II from normal subjects will be continued during the coming year. The structure of normal apoC-II will be of major importance in our understanding of the structure-function requirement of lipoprotein lipase activation by apoC-II. Selected proteins and peptides will be synthesized by the solid phase technique. The development of improved techniques for solid phase synthesis will make possible detailed structure-function studies on plasma apolipoproteins. The continued elucidation of the structure and function of plasma apolipoproteins will be a prerequisite to our ultimate understanding of the molecular mechanisms involved in lipid transport, metabolism, and atherosclerosis.

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02011-07 MDB

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Molecular Properties of Lipoproteins and Apolipoproteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH

Molecular Disease Branch

SECTION

Peptide Chemistry

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.0	2.0	4.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

These projects are directed towards a greater understanding of the quaternary organization of plasma lipoproteins and of the function of the oligomeric species involved in the transport and metabolism of lipids in plasma. The apolipoprotein composition of plasma lipoproteins is viewed as the governing factor in directing lipoprotein metabolism. Specificity is believed to be related directly to apolipoprotein secondary, tertiary and quaternary structure. Studies of the molecular properties of plasma lipoproteins have been extended to include lipoprotein lipase. Purification of lipoprotein lipase and hepatic lipase from human post-heparin plasma has been initiated. Studies of the molecular properties of radiolabeled apolipoproteins have been extended to include in vivo metabolic studies with apolipoprotein A-I. A knowledge of the specific interactions and quaternary organization of plasma lipoprotein complexes is fundamental to our ultimate understanding of lipid metabolism.

754

PHS-6040
(Rev. 2-81)

Project Description:

Objective:

1) Purification of enzymes involved in the metabolism of plasma lipoproteins; lipoprotein lipase and hepatic lipase:

Human post heparin plasma contains two major lipases, lipoprotein lipase (LPL) and hepatic lipase (HL). LPL is associated with extrahepatic cells and is believed to play a major role in the hydrolysis of triglycerides in chylomicrons and VLDL. Triglyceride hydrolysis by LPL may be increased several fold in the presence of a specific activator, apolipoprotein C-II. Thus a deficiency in LPL or apoC-II may lead to the fasting chylomicronemia and mildly elevated VLDL which is characteristic of type I hyperlipoproteinemia. The metabolic role of HL is less well characterized. This enzyme is believed to be associated with hepatic tissue in vivo and has been postulated to play a role in HDL as well as VLDL phospholipid and triglyceride metabolism. The enzymic hydrolysis of triglycerides by HL can be increased several fold in vitro by the addition of apolipoprotein A-II. This activation has many of the characteristics of the apoC-II activation of LPL.

Although LPL and HL have many common features, they are clearly different enzymes and can be physically as well as immunochemically separated from one another. Clinical abnormalities of the lipase system can be classified and characterized by quantitating the LPL and HL enzymic activity of pre- and post-heparin plasma; both HL and LPL are released into the circulation with the injection of heparin. The most efficient way to quantitate these activities is to employ specific antibodies to HL and LPL in the assay system. We have begun the purification of LDL and HL in order to develop specific antibodies to aid in our evaluation of patients with abnormalities of the lipase system. In addition, the molecular properties of the purified enzymes shall be compared in order to understand more fully the similarities and differences between HL and LPL.

Major Findings:

LPL and HL purification scheme.

Human post-heparin plasma, obtained after injection of 75 U heparin per kg body weight, is diluted with an equal volume of buffer A (5 mM sodium barbital, 0.4 M sodium chloride, 0.001 M sodium azide, pH 7.5) and allowed to stir overnight at 4°C with a sepharose resin to which heparin has been covalently attached. The following day the resulting heparin-sepharose gel is transferred to a sintered glass funnel and washed with buffer A until the optical density of the eluate is below 0.1 (280 nm). A column of the washed heparin-sepharose is prepared and washed with an additional 100 ml of buffer A (rate 37 drops/min). HL is then eluted with buffer B (5 mM sodium barbital, 0.8 M sodium chloride, 0.001 M sodium azide, pH 7.5) and LPL is eluted with buffer C (5 mM sodium barbital, 1.5 M sodium chloride, 0.001 M sodium azide, pH 7.5). The fractions containing HL and LPL are pooled and dialysed separately against buffer D (3.6 M ammonium sulfate 0.01 M potassium phosphate pH 6.5). The resulting precipitates are then dissolved in 50% (v/v) glycerol, 0.01 M potassium phosphate pH 7.5 buffer. From 500 ml of

post-heparin plasma 2 ml of enzyme in the glycerol-phosphate buffer are obtained with overall yields of 65% for LPL and 75% for HL. The resulting partially purified enzymes are then fractionated using high performance liquid chromatography and a TSK-3000 column matrix. In 0.01 M Tris, 0.1 M sodium chloride 0.001 M sodium azide pH 7.4 buffer both enzymes bind to the column matrix; activity is found after the salt volume and yield for both enzymes is quite poor. In the presence of imidazole at pH 6.5 both enzymes elute within the column volume with yields of 35% for HL and 25% for LPL. SDS gel electrophoresis shows two major bands for HL and 3 major bands for LPL at this stage of purification.

Objective:

2) Evaluation of the molecular properties of enzymes involved in lipoprotein metabolism; studies on bovine lipoprotein lipase.

Lipoprotein lipase is an enzyme which hydrolyses the triglyceride and phospholipid components of plasma lipoproteins. In the presence of a specific activator, apolipoprotein C-II, triglycerides are the preferred substrate and lipoprotein lipase is believed to be the primary enzyme responsible for hydrolysis of the triglyceride components of VLDL and chylomicrons. Characterization of the physical properties of lipoprotein lipase has been hampered by the instability of the purified enzyme. At low salt concentrations lipoprotein lipase is virtually insoluble in the absence of anionic detergents. Iverius and Ostlund-Lindquist (1976) found it impossible to carry out sedimentation equilibrium studies at physiological ionic strangth because of rapid self-aggregation of the enzyme. High salt concentration, i.e., 1.5 M NaCl, as used in the present study, decreases the tendency of the enzyme to self-aggregate. In this medium solutions of up to about 1 mg of protein per ml are usually rather stable below 10°C; in our experiments less than 10% of the enzymic activity was lost during storage at 4°C for up to one week. At present, running the experiments in this type of buffer and at low temperatures is the only system in which the physical properties of catalytically active lipoprotein lipase can be studied. The enzyme is active and can be stimulated by apolipoprotein C-II in this system.

We have also evaluated the molecular properties of trypsin-treated lipoprotein lipase. It has been shown previously that bovine lipoprotein lipase can be cleaved by trypsin into two pieces of similar size which are held together by disulfide bonds. After this cleavage the enzyme retains virtually full activity against simple substrates, such as p-nitrophenylacetate, but its activity against emulsions of long-chain triglycerides is much reduced. The tentative interpretation of this previous work was that tryptic cleavage disrupted the cooperation between the domain on the enzyme responsible for lipid interaction and the region which contains the active site. Over the past year we have investigated the molecular size and conformation of native and trypsin-treated lipoprotein lipase in order to characterize more fully this unique system.

Methods Employed:

Secondary structure, i.e., α-helical, β-pleated sheet, and random configurations, was investigated by using a Cary model 61 spectrometer. Tertiary structure, i.e., the mode of intramolecular folding, was investigated by monitoring changes in the fluorescence and absorption properties on a Perkin-Elmer MPF-4 fluorescence spectrophotometer and a Beckman Acta 3 spectrophotometer.

Quaternary structure, i.e., intermolecular protein interaction, was studied by molecular weight measurements with a Spinco Model E ultracentrifuge and a Chromatrix Laser Light Scattering Photometer. Calculations and least square fits of the data to various models were performed with the MLAB program on the DEC system 10 computer at NIH.

Major Findings:

The molecular size of monomeric lipoprotein lipase before and after treatment with trypsin was estimated by gel permeation chromatography in 6 M guanidinium chloride 0.01 M Tris, 0.1 M NaCl pH 7.4. The column support used was TSK 3,000 SW gel available from Toyo Soda, Tokyo, Japan. The elution volume of known standards in this buffer was a linear function of the logarithm of molecular weight. Lipoprotein lipase eluted as a symmetrical peak under these conditions; the elution volume corresponding to a molecular weight of 47,500. Approximately 90% of trypsin treated lipase also eluted in this position, i.e., v_e = 12.1 ml; the remainder eluted as several peaks in the more included portion of the column. The nature of the low molecular weight material is not known; we have been unable to remove these fragments in non-denaturing buffers.

The monomer molecular weight of bovine lipoprotein lipase before and after treatment with trypsin was determined directly by sedimentation equilibrium measurements in 6 M guanidinium chloride at several rotor speeds. The untreated enzyme behaved as a homogeneous species at all rotor speeds. In order to establish the molecular weight unequivocally, we also determined partial specific volumes in 6 M guanidinium chloride by comparison of equilibrium profiles of concentration versus radius in buffers prepared with H_2O and D_2O. The value obtained, 0.71 ml/g, is in agreement with that predicted from amino acid and carbohydrate composition. With this value the molecular weight of native lipoprotein lipase is 41,700. The corresponding value for trypsin-treated material, assuming the same partial specific volume, was 41,500 at low rotor speeds, i.e., 15,000 rev./min. This value decreased to 35,400 at 30,000 rev./min., indicating the presence of low molecular weight species in the trypsin treated material. The data at both rotor speeds were analyzed simultaneously in order to estimate the amount of low molecular weight species present in the original sample. Assuming a single low molecular weight species, the data were most consistent with the presence of 3 wt % of a 20,000 molecular weight species. Thus 97 wt % of the trypsin-treated material used in these studies had a molecular weight equivalent to native lipoprotein lipase.

For studies of the physical properties of the native enzyme we used a buffer containing a high concentration of inorganic salt; 1.5 M NaCl, 10 mM Tris, pH 7.4. Purified lipoprotein lipase is only sparingly soluble at lower ionic strengths. In this buffer limiting weight average molecular weights of 80,000 and 174,000 were obtained by sedimentation equilibrium, which is consistent with the presence of dimers and higher molecular weight oligomers under these conditions. For the trypsin treated enzyme, limiting weight average molecular weights of 30,000 and 100,000 were obtained by sedimentation equilibrium. These lower values as compared to native lipase may be due to the presence of low molecular weight fragments in the trypsin treated material.

Circular dichroic measurements were performed in 10 mM Tris-Cl, 1.5 M sodium chloride, pH 7.4 at 8°C. The native enzyme exhibited a broad negative maximum between 220 and 205 nm characteristic of globular proteins. Tryptic treatment resulted in a time dependent loss of ellipticity. At four hours the tryptic cleavage was virtually complete; time dependent losses of secondary structure had reached a plateau and less than 5% remained of the activity measured against Intralipid^{R} activated by human serum. On the other hand 87% of the activity against p-nitrophenylacetate remained.

Trypsin treatment resulted in major decreases in mean residue ellipticity between 205 and 235 nm; the shape of the difference spectra resembled that expected for residues in a helical conformation. We have now shown that trypsin treatment results in a conformational change in the enzyme molecule, presumably due to creation of metastable states that have a lower free energy than those available to native lipase. The distribution of enzyme between these states would be expected to depend critically upon environment. However, the conformational changes apparently do not directly involve the active site; hydrolysis of soluble substrates such as p-nitrophenylacetate is almost unaffected by tryptic cleavage. It has been postulated that lipoprotein lipase exists in different molecular/catalytic forms at lipid-water interfaces, with apolipoprotein C-II acting by stabilizing the most active form; the distribution of enzyme between these forms would depend upon the quaternary organization of the interface.

The presence of less active or inactive molecules, which are activatable by apolipoprotein C-II, is consistent with minor reversible conformational changes upon adsorption to lipoprotein particles. The observed loss of secondary structure upon treatment with trypsin, which presumably leads to a more flexible molecule, could allow increased unfolding (refolding to a different conformation) on the surface of the particle. The efficiency of substrate hydrolysis in a given environment would depend upon the ease with which substrate or effectors could generate the most active conformation of the enzyme.

3) Objective:

Automation of data collection and analysis for the Cary Model 61 Spectropolarimeter.

In our evaluation of the structure-function relationships for the proteins and enzymes involved in lipoprotein metabolism we have depended heavily upon the correlation between changes in secondary structure and molecular weight. Molecular weights are now obtained routinely in my laboratory with a Model E ultracentrifuge which is interfaced with an automated data collection system. The rate limiting step in comparing molecular weight and secondary structure is the collection and calculation of circular dichroic data. An automatic method of collecting and calculating circular dichroic data would minimize operator error and increase greatly the efficiency of my laboratory.

Major Findings:

In collaboration with Ramon L. Tate and Arthur R. Schultz, Jr. (DCRT) the Cary Model 61 Spectropolarimeter was interfaced with a PDP 11/03 microcomputer. A simple, flexible CD spectropolarimeter/ microcomputer interface design and an

interactive data processing program system was developed. Apolipoprotein CD spectra may be acquired, averaged, subtracted, converted to mean residue ellipticities, printed and stored for future use. Stored data may also be conveniently transferred to a large computer facility for semi-automatic conformation analysis. With this system usable CD measurements of apoC-I were obtained at protein concentrations as low as 2.5 ug/ml at a path length of 1 cm. The combined system is efficient and minimizes operator error in evaluation of the secondary structure of proteins.

Objective:

4) Evaluation of the molecular properties of apolipoproteins.

The composition of plasma lipoproteins is dependent, through the laws of mass action, upon the composition and concentration of other components of plasma. The apolipoprotein composition of plasma lipoproteins directs the course of lipoprotein metabolism. Thus newly secreted VLDL gain a complement of apolipoproteins that govern the activation/inhibition of enzymic lipid hydrolysis and/or uptake by specific receptors on cells. With metabolism the lipoprotein particles are modified covalently, changing their affinity for specific apolipoprotein and/or lipoprotein complexes, which results in a redistribution of apolipoproteins. The role that lipid-free species play in these exchange/transfer processes is not known. In order to quantitate these interactions we have undertaken a systematic investigation of the solution properties of apolipoproteins. Previous reports have stressed the self-association of apolipoproteins in aqueous solution with particular emphasis on the major conformational changes that are concomitant with association.

In their monomeric form, apolipoproteins are loosely folded and have a high degree of exposure of non-polar residues to solvent. Presumably the amino acid sequence of apolipoproteins is such that there is little gain in free energy upon sequesting non-polar groups from solvent; counteracting forces are the maintenance of all hydrogen bonds and the loss of entropy in the monomeric folded gobular form. Non-polar groups are sequested from solvent by self- and mixed associations and interaction with other non-polar compounds. The affinity for these interactions is quite high at neutral pH and physiological ionic strength and it is difficult to evaluate, experimentally, the conformation of monomeric apolipoproteins. During the past year we have extended circular dichroic measurements of apoC-I to much lower protein concentrations in order to examine the folding of the monomeric species.

Methods Employed:

Secondary structure, i.e., α-helical, β-pleated sheet, and random configurations, was investigated by using a Cary model 61 spectropolarimeter. Tertiary structure, i.e., the mode of intramolecular folding, was investigated by monitoring changes in the fluorescence and absorption properties on a Perkin-Elmer Model MPF-4 fluorescence spectrophotometer and a Beckman Acta 3 spectrophotometer. Quaternary structure, i.e., intermolecular protein interaction, was studied by molecular weight measurements with a Spinco Model E ultracentrifuge and a Chromatrix Laser Light Scattering Photometer. Calculations and least square fits of the data to various models were performed with the MLAB program on the DEC system 10 computer at NIH.

Major Findings:

Lipid-free apoC-I self-associates with major increases in secondary structure upon oligomer formation. Monomeric apoC-I is believed to be loosely folded, resembling a random coil. Measurements of the secondary structure of monomeric apoC-I have been precluded in the past due to the low signal/signal+noise ratios at concentrations of protein required for appreciable dissociation of oligomeric apoC-I. Circular dichroic (CD) studies of human apoC-I were performed using a complete computerized data acquisition and processing system interfaced with a Cary 61 spectropolarimeter. With this set-up we examined the circular dichroic spectra of low concentrations of apoC-I; these data were not previously obtainable because of a low signal/noise ratio. At the lowest concentration investigated, 0.25 μg/ml the circular dichroic spectra of apoC-I resembles that expected of a random coil. At higher concentrations approximately 65% of the amino acid residues of apoC-I are in an α-helical configuration. This represents a major refolding of the polypeptide backbone with self-dissociation alone, which is comparable to the temperature, pH or guanidinium chloride unfolding of gobular proteins. Thus for this system the stability of the folded species can be evaluated experimentally without using extremes in temperature, pH or denaturants such as guanidinium chloride. The ease with which monomeric apoC-I can accomodate a random configuration as compared to gobular proteins, is presumably related to the ease with which apoC-I can rearrange and accomodate other environments such as the surface of lipoprotein particles. This flexibility may account for the rapid exchange/transfer of apoC-I between lipoprotein particles.

Objective:

5) Evaluation of the molecular properties of radiolabeled apolipoproteins.

Radiolabeled apolipoproteins are routinely used to evaluate the *in vivo* metabolism of plasma lipoproteins. A basic assumption in these studies is that the labeled apolipoprotein mimics the distribution and metabolism of the unlabeled species. Comparison of the properties of radiolabeled and native species requires a thorough knowledge of the solution properties of the apolipoprotein in question. The molecular properties of several apolipoproteins have been sufficiently well characterized to allow a quantitative comparison between labeled and unlabeled species. As summarized in last years annual report we could not detect any differences in the molecular properties of radiolabeled and native apolipoprotein A-II. In contrast, iodination of apoA-I results in the formation of an incompetent monomer due to either double labeling of a single protein molecule or the labeling of a specific tyrosine residue. We were able to separate the labeled species into two pools, one of which was indistinguishable from the native unlabeled species. Those results were based on *in vitro* solution properties of the isolated apolipoprotein and the two pools may have the same kinetics and fate with *in vivo* metabolic studies. If this were the case then unfractionated radiolabeled apoA-I could be used with confidence, otherwise the labeled species would have to be fractionated prior to metabolic studies. During the past year we have compared the kinetics of *in vivo* metabolism of competent and incompetent radiolabeled apolipoprotein A-I.

Major Findings:

In collaboration with Dr. E.J. Schaefer apolipoprotein A-I was radiolabeled with ^{125}I and ^{131}I using iodine monochloride. The resulting apolipoproteins were then fractionated separately, using high performance liquid chromatography, into competent (pool 1) and incompetent (pool 2) species. A TSK-3000 column was used for fractionation and the entire procedure took less than 1 hour to complete. ^{131}I labeled pool 1 and ^{125}I labeled pool 2 were selected for further studies and were mixed and injected simultaneously into two normal volunteers. Samples of plasma (10 min, 6 h, 12 h and daily) and cumulative urine were then collected for 7 days. The incompetent monomer (^{125}I labeled pool 2) decayed more rapidly from plasma than the competent monomer (^{131}I labeled pool 1) and appeared more rapidly in the urine of both subjects. Therefore, these two pools of apolipoprotein A-I behave differently in vivo as well as in vitro and this should be taken into account in future metabolic studies. A detailed analysis of the kinetics of metabolism of these two pools is currently underway.

Objective:

6) Evaluation of the mechanism of regulation of enzymes involved in lipid metabolism: studies on hepatic lipase.

Hepatic lipase (HL) and lipoprotein lipase (LPL) account for the majority of the lipase activity in human post heparin plasma. LPL plays an important role in the metabolism of triglyceride-rich lipoproteins and its deficiency results in type I hyperlipoproteinemia with fasting chylomicronemia. The mechanism of regulation of HL is not completely known. The addition of serum to assay medium has been shown to activate the HL hydrolysis of gum-arabic-triolein emulsions whereas apolipoproteins C-I, C-II, and C-III inhibit HL activity. It has also been reported that HL binds to HDL resulting in the inhibition of hydrolysis of oleic acid-labeled intralipid. During the course of our investigations of HL we found that apolipoprotein A-II increases the in vitro enzymic activity of HL by 3.6 fold. Over the past year we have extended these studies to include the effects of temperature, pH and assay conditions. In addition, the effect of normal plasma, plasma lipoproteins, and apolipoproteins A-I, C-I, C-II, and C-III on hepatic enzymic activity were investigated.

Major Findings:

We have demonstrated previously that the plasma activation of HL mediated triglyceride hydrolysis in vitro using a phospholipid-triolein-glycerol emulsion is due to apoA-II, a major HDL component. ApoA-II stimulated HL when added in μmolar concentrations, and this effect was specific for apoA-II since all other HDL apolipoproteins studied (apoA-I, apoC-I, apoC-II, and apoC-III) were inhibitory. The activation is seen at 25°C as well as 37°C and observed in the pH range of pH 7.5-9.5.

When added 10 min after starting triglyceride hydrolysis by HL, isolated apoA-II increased HL mediated triglyceride hydrolysis immediately to a rate similar to the rate obtained when added before starting triglyceride hydrolysis. This demonstrates that the activation caused by apoA-II is not due to the stabilization of a fully active enzyme that becomes inactivated during the course of the assay.

Our results indicate that the physical state of an assay system using the phospholipid stabilized triolein-emulsion is such that the addition of apoA-II to the assay could in fact cause a decrease in the amount of triolein hydrolized by HL if one did not provide adequate mixing of the reagents. It is thus apparent that the stimulating effect of apoA-II is very sensitive to the structure of the substrate and we would expect that it will also be sensitive to the composition of the substrate. The composition and structure of the substrate is also known to be important in the apoC-II activation of LPL.

Recombined particles increased HL activity when apoA-II was used in the protein moiety whereas recombined apoC-III caused inhibition. The higher concentration needed for full activation of HL as compared to isolated apoA-II could be accounted for by postulating a redistribution of apoA-II between the recombined particle and the phospholipid-triglyceride substrate. Similarly, the lesser degree of HL inhibition observed with the apoC-III may be due to a similar competition between the recombined particle and substrate.

The combined data provide evidence that apoA-II regulates the in vitro enzymic activity of HL as an apolipoprotein, in recombined particles, and as a constituent of HDL. The in vitro activation of HL by apoA-II parallels the apoC-II activation of lipoprotein lipase on triglyceride emulsions.

Significance to Biomedical Research and Program of the Institute:

These investigations are directed toward a greater understanding of the quaternary organization and function of the plasma lipoproteins. The apolipoprotein and lipid composition of plasma lipoproteins is related directly to the concentration and composition of other components of plasma, including other plasma lipoproteins. Enzymic hydrolysis of lipids in plasma is mediated through apolipoprotein effectors which distribute among plasma lipoproteins according to the laws of mass action. A quantitative knowledge of these types of interactions is fundamental to our understanding of lipid transport and metabolism in normal individuals and in patients with disorders of lipid metabolism and atherosclerosis.

Proposed Course:

Investigations concerning the molecular properties of apolipoproteins shall be continued. A major emphasis shall be the purification of human lipoprotein lipase and hepatic lipase and the production of specific antibodies for these enzymes. Studies on effectors of lipase activity shall be extended to include other apolipoproteins. These studies continue to form a framework for our ultimate understanding of in vivo plasma lipoprotein interactions.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 02012-07 MDB

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Properties and Regulation of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Zafarul H. Beg, Ph.D.	Research Chemist	MDB	NHLBI
Other:	John A. Stonik, B.S.	Chemist	MDB	NHLBI
	James C. Osborne, Jr., Ph.D.	Research Chemist	MDB	NHLBI
	H. Bryan Brewer, Jr., M.D.	Chief	MDB	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Molecular Disease Branch

SECTION

Peptide Chemistry

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The molecular properties of purified rat liver HMG-CoA reductase (HMGR) have been investigated. A molecular weight of 320,000 was obtained for holoenzyme by sedimentation equilibrium and high performance liquid chromatography (HPLC). The purified enzyme migrated as two bands on analytical slab-SDS-gel electroporesis. The monomer molecular weights by sedimentation equilibrium for upper and lower bands were 54,000 and 52,000 respectively. Purified HMGR can be separated into active and inactive forms by sucrose density gradient centrifugation. On slab-SDS-gel electrophoresis the upper (54,000) and lower (52,000) bands were derived from inactive and active forms of HMGR. Under different physiological situations the ratio of active and inactive forms of HMGR shifted dramatically. The interconversion of two species of HMGR may represent an additional mechanism for the in vivo regulation of cellular cholesterol biosynthesis. The cytosolic reductase kinase (RK) and mevalonate kinase (MK) have been separated by three different techniques. Fractions containing only RK reversibly inactivated HMGR. Fractions containing only MK or mixture of MK and RK revealed artifactual RK activity in the absence of EDTA or mevalonate; however addition of EDTA or mevalonate before HMGR assay eliminated any apparent decline in HMGR activity.

Project Description:

Objective:

1) Molecular weight determination of rat liver HMGR.

Methods Employed:

i) Gel Permeation Chromatography:

Gel permeation chromatography was performed with Sephadex G-150 superfine or Sepharose 6B in 0.05 M KCl, 0.04 M KH_2PO_4, 0.03 M EDTA, 0.1 M sucrose, 0.005 M DTT, 0.001 M sodium azide, pH 7.2 (buffer A) at room temperature. The column (90 X 1.5 cm) was equilibrated with buffer A, the flow rate was 6.8 ml/h and .5 ml fractions were collected in a LKB fraction collector. Elution profiles were obtained by absorbancy at 280 nm, using a Beckman Acta III spectrometer and HMG-CoA reductase activity was determined. The proteins used for column calibration, ferritin (mr = 540,000), catalase (mr = 240,000), aldolase (mr = 158,000), bovine serum albumin (mr = 68,000), chymotrypsinogen (mr = 25,600), and cytochrome C (mr = 12,500), were purchased from Boehringer Mannheim Biochemicals.

ii) Sucrose Density Gradient Centrifugation:

The molecular weight of crude and purified HMG-CoA reductase from sucrose density gradient centrifugation was determined as described by Martin and Ames, using 5 to 20 percent sucrose gradients in buffer B (0.05 M KCl, 0.04 M KH_2PO_4, 0.03 M EDTA, 2 mM DTT, pH 7.0). Molecular weights were determined by sedimentation equilibrium utilizing the Beckman Model E ultracentrifuge. Data were collected automatically with a computer acquisition system. The subunit molecular weight of HMG-CoA reductase was obtained in 6 M guanidine hydrochloride.

iii) Airfuge Centrifugation:

Molecular weight determinations of crude or purified HMG-CoA reductase by measuring enzymic activity were performed in a Beckman Spinco airfuge in the cold at 4°C. HMG-CoA reductase samples were dissolved in 100 microliters of buffer A containing bovine serum albumin to attain a 0.5 percent total protein concentration in order to provide a stabilizing density gradient, and centrifuged in a cellulose nitrate tube (175 microliter capacity). Centrifugation was continued for 24 h, after which successive 10 microliter samples were carefully withdrawn with a Hamilton syringe. Each fraction was diluted and analyzed for HMG-CoA reductase activity and total protein.

iv) High Performance Liquid Chromatography (HPLC):

Partially purified or homogenous preparations of HMG-CoA reductase were analyzed by HPLC using a Beckman model 332 HPLC equipped with a model 160 uv-visible detector and a Fisher series 5000 redorder. The column, an Altex Spherogel (TSK-3000-SW, 7.5 mm x 30 cm), was equilibrated at room temperature with a buffer containing 50 mM KCl, 40 mM potassium phosphate, 30 mM EDTA, pH 7.2, 1 mM DTT, 100 mM sucrose, and operated at a flow rate of 0.3 ml/min. Enzyme

fractions eluted in the above buffer (250 microliter fractions) were detected by absorbancy at 280 nm. HMG-CoA reductase activity was assayed in each fraction. The column was calibrated by using ferritin (mr = 540,000), catalase (mr = 240,000), aldolase (mr = 158,000), and bovine serum albumin (mr = 68,000).

Major Findings:

The molecular weight of purified phosphorylated and dephosphorylated HMG-CoA reductase was determined on Sephadex G-150 columns. When phosphorylated or dephosphorylated HMG-CoA reductase was applied to a Sephadex G-150 column, the enzyme activity and protein peaks co-eluted. A molecular weight of approximately 320,000 was estimated for both forms of HMG-CoA reductase. However, the elution profile and molecular weight of phosphorylated HMG-CoA reductase was slightly higher in comparison to the dephosphorylated form. Inclusion of 0.5 M KCl in buffer A did not affect the apparent molecular weight. The molecular weight of HMG-CoA reductase was also determined by using sedimentation equilibrium. Purified HMG-CoA reductase was analyzed in the absence or presence of 6 M guanidinium chloride. The deviation was a random function of radius which is further evidence of homogeneity of the sample. The average molecular weight for six different preparations of HMG-CoA reductase was 323,152 $\pm$ 7,000.

The molecular weight of soluble or partially purified HMG-CoA reductase by sucrose density gradient centrifugation was 104,000. However, when fractions from purified enzyme were analyzed for protein content, two protein peaks were observed. The first protein peak was associated with little enzyme activity (inactive form), and corresponded to an apparent molecular weight of 320,000 when analyzed by gel permeation chromatography of HPLC. The second peak coincides with most of the detectable HMG-CoA reductase activity (active form) with an apparent molecular weight of 104,000. Sucrose density gradient fractionation has enabled us to identify two apparently different molecular forms of HMG-CoA reductase. On slab SDS-gel electrophoresis the upper (54,000) and lower (52,000) bands were derived from inactive (320,000) and active (104,000) species of HMG-CoA reductase respectively. The molecular weight of the faster migrating species obtained from sucrose gradient centrifugation when analyzed by gel permeation chromatography on Sephadex G-150 eluted as a 320,000 molecular weight species. Moreover, on gel filtration, the HMG-CoA reductase activity and protein of the dissociated lower molecular weight species (104,000) obtained by sucrose density centrifugation also eluted as a single peak corresponding to an apparent molecular weight of 320,000.

Determination of the molecular weight of HMG-CoA reductase by airfuge centrifugation confirmed the findings of sucrose density gradient centrifugation. Analysis of crude or partially purified HMG-CoA reductase by this technique gave a molecular weight of 104,000. However, when both protein and enzymic activity of purified HMG-CoA reductase was analyzed a lower (104,000) and a higher (320,000) molecular form was obtained. These results demonstrate that airfuge or sucrose gradient centrifugation dissociate HMG-CoA reductase into two distinct forms.

We have also separated the active and inactive forms of HMG-CoA reductase by high performance liquid chromatography (HPLC) utilizing TSK-3000-SW columns. Analysis of inactive and active peaks of HMG-CoA reductase by HPLC in the presence of 6 M guanidinium chloride revealed molecular weights of 54,000 and 52,000

respectively. These results are consistent with sucrose density gradient centrifugation data. The amount of inactive HMG-CoA reductase was higher in preparations with low specific activity, and significantly lower in samples with high specific activity.

Objective:

2) In vivo interconversion of inactive and active forms of HMGR.

Methods Employed:

Male Sprague-Dawley rats weighed 225-250g at the time of killing, were used for the purification of HMG-CoA reductase. The rats were maintained for 10 days in a light-controlled room (dark, 2:30 am to 2:30 pm). The animals had free access to food and water. The rats where indicated were fed a diet of ground rat chow containing 3 percent cholestyramine. For diurnal high and low experiments rats were killed at 8:30 am and 8:30 pm respectively. Cholestyramine fed rats were also killed at 8:30 am.

HMGR was purified to homogeneity utilizing liver microsomes from chow-fed rats killed at diurnal low, diurnal high, and cholestyramine fed rats at diurnal high. The purified HMGR was analyzed by HPLC and fractions were used for activity determinations and SDS-gel electrophoresis.

Major Findings:

The ratio of active and inactive forms of HMGR shifted dramatically under different physiological situations. At the nadir of diurnal variation most of the HMGR was in the inactive form. At the peak of diurnal rhythm HMGR has a mixture of active and inactive forms. Following cholestyramine feeding almost all of the inactive form has shifted to the active species. These results establish that inactive HMGR protein as convertible to the active form in in vivo. All attempts to reactivate inactive HMGR in vitro have failed.

The associated mixture of HMG-CoA reductase had a molecular weight of 320,000 on HPLC, however the inactive form eluted earlier than the active species. The apparent molecular weight of the subunit of the faster migrating component on HPLC was 54,000 by slab SDS gel electrophoresis. The slower migrating component, which was associated with the majority of enzyme activity also gave a single band upon slab gel electrophoresis which corresponded to a molecular weight of 52,000.

The combined results are consistent with the existence of a new mechanism for the regulation of HMG-CoA reductase. The in vivo interconversion of these two forms of HMGR may represent an important physiological event in the regulation of cellular cholesterol biosynthesis. Since inactive HMGR can not be dephosphorylated (activated) in vitro by phosphoprotein phosphatase, the in vivo regulation of the two forms of reductase reported here represent a new mechanism for the rapid regulation of cholesterol biosynthesis in cells.

Objective:

3) Reductase kinase dependent inactivation of HMGR: Separation of mevalonate kinase and reductase kinase.

Methods Employed:

i) Purification of Microsomal HMG-CoA Reductase: Rats fed 3% cholestyramine (5 days) were killed at the mid-dark period. Microsomes were isolated and used for solubilization and purification of HMG-CoA reductase.

ii) Isolation of Reductase Kinase-free Microsomal HMG-CoA Reductase: The unwashed microsomal pellet was suspended in a buffer containing 50 mM potassium phosphate, pH 7.2, 250 mM KCl, 2 mM DTT, and 10% glycerol and incubated at 37°C for 30 min and stored frozen at -20°C. Microsomal HMG-CoA reductase was not inactivated by ATP-Mg when subjected to the above treatment. The preparation was completely devoid of reductase kinase and reductase kinase kinase. HMG-CoA reductase was stable for at least one year if stored frozen.

iii) Isolation of Cytosolic Reductase Kinase: Livers were homogenized in 50 mM KH_2PO_4, pH 7.4, 250 mM NaCl, 1 mM EDTA and 5 mM DTT (ESDP buffer) containing 50 mM NaF and 100 um phenylmethylsulfonyl fluoride (PMSF). The high speed (100,000 x g) supernatant was carefully removed and stored at -20°C. Phosphoprotein phosphatase was purified from the cytosolic fraction which was isolated in 50 mM imidazole, pH 7.4, 250 mM NaCl, 1 mM EDTA and 5 mM DTT (ESDI buffer).

iv) Assay of Reductase Kinase Activity: Purified HMG-CoA reductase or microsomes that retained HMG-CoA reductase activity but no reductase kinase activity were used to determine the activity of cytosolic reductase kinase. The first step in the assay involved preincubation of HMG-CoA reductase in duplicate with reductase kinase at 28°C (30 min) in a medium containing 50 mM β-glycerophosphate (pH 7.0), 5 mM DTT, 50 mM NaF, 10 mM magnesium acetate, 4 mM ATP, and 20 mg/ml BSA in a total volume of 200 µl. Control tubes contained all the ingredients except ATP. At the end of the preincubation time, the reaction was stopped by the addition of EDTA (pH 7.0, 30-fold excess in relationship to Mg^{2+} concentration). The second step involved the assay of residual HMG-CoA reductase activity in a total volume of 400 µl at 37°C for 10-15 min. The reaction was started by the addition of 0.15 M KH_2PO_4 (pH 6.9) buffer containing 0.2 M KCl, 10 mM mevalonic acid, 0.75 mM NADPH and .0375 mM DL-[3-^{14}C]-HMG-CoA (5 µci/umole). The reaction was terminated by the addition of 50 ul of 10 N HCl. Tubes were centrifuged and 200 µl of aliquots were applied to 1 ml columns of Bio-Rex 5 resin. ^{14}C-mevalonolactone was eluted with 2 ml of water, 15 ml of aquasol was added and the resulting mixture was assayed for radioactivity. One unit of reductase kinase is defined as the amount which causes a decline of 1 unit of HMG-CoA reductase during a 30 min preincubation. One unit of HMG-CoA reductase catalyzes the formation of one pmol of mevalonate in 1 min under standard assay conditions.

v) Assay of Mevalonate Kinase Activity: Cytosol or fractions of cytosol were assayed for mevalonate kinase activity. Reaction mixtures contained 50 mM 4-(2-hydroxy-ethyl)-1-piperazineethane sulfonic acid (pH 7.4), 2 mM ATP, 4 mM $MgCl_2$, 2 mM DTT, 0.67 mM [^{14}C]-RS-mevalonate (.121 uCi/umole), and varying aliquots of enzyme. The reaction was initiated by the addition of [^{14}C]-RS-mevalonate.

Following incubation at 37°C for 10-15 min, the reaction was terminated by the addition of 10 µl of 2.4 N HCl. The reaction mixture was maintained at 37°C for 30 min to ensure complete lactonization. The [^{14}C]-phosphomevalonate was separated on Pasteur pipette columns (1.5 ml) containing Bio-Rad AG1-X8 resin. The columns were washed sequentially with 6 ml of 0.01 N HCl, and 4 ml of 2.4 N HCl which eluted the phosphomevalonate. One unit of mevalonate kinase catalyzes the formation of one nmol of phosphomevalonate in 1 min.

Major Findings:

Reductase kinase is a cytosolic enzyme which inactivates (phosphorylates) HMG-CoA reductase in the presence of ATP-Mg^{++}. The inactivation was completely reversed by phosphoprotein phosphatase. Cytosol or its fractions or extracts of unwashed microsomes used for reductase kinase also contain mevalonate kinase. Incomplete complexation of ATP-Mg or removal of mevalonate kinase following inactivation of HMG-CoA reductase by ATP-dependent reductase kinase would permit mevalonate kinase to consume the [^{14}C]-mevalonate formed from [^{14}C]-HMG-CoA during the HMG-CoA reductase assay. The loss of [^{14}C]-mevalonate can be completely blocked by the addition of either a 30-fold excess of EDTA (in relationship to Mg^{++}) or 10 mM mevalonate before starting the HMG-CoA reductase assay.

In the absence of EDTA or mevalonate, cytosol revealed artifactual reductase kinase activity due to the loss of [^{14}C]-mevalonate due mevalonate kinase activity. Addition of excess EDTA or mevalonate was associated with authentic reductase kinase activity. Furthermore, cytosolic mevalonate kinase activity was detected only in the absence of EDTA or mevalonate. These results support the conclusion that reductase kinase and mevalonate kinase are two different enzymes. In order to definitively establish that reductase kinase and mevalonate kinase are separate enzymes, two kinases have been separated by three purification schemes. Cytosol or fractions of cytosol were fractionated by: a) chromatography on DEAE sephacel; b) ammonium sulfate; and c) chromatography on 5% agarose-Procion Red HE3B. Mevalonate kinase eluted in the void volume whereas reductase kinase was bound to DEAE sephacel and eluted with 0.5 M KCl. The washed (4x) 0-25% ammonium sulfate fraction of cytosol was associated with reductase kinase activity with less than 1% mevalonate kinase activity. Most of the mevalonate kinase activity remained in the post 25% supernatant. Reductase kinase at pH 6.5 did not bind to a 5% agarose-Procion Red HE3B column, whereas mevalonate kinase bound to the column and was eluted with ESDP buffer. Less than 5% of cytosolic mevalonate kinase activity was present in the void volume.

The fraction containing only mevalonate kinase activity was associated with artifactual reductase kinase activity due to phosphorylation of the [^{14}C]-mevalonate formed during HMG-CoA reductase assay when assayed in the absence of EDTA or mevalonate. Artifactual reductase kinase activity was completely abolished by the inclusion of excess EDTA or mevalonate in the incubation prior to HMG-CoA reductase assay. The fractions containing authentic reductase kinase activity with no mevalonate kinase activity inhibited HMGR equally either in the presence or absence of EDTA or mevalonate. The prerequisite for the expression of mevalonate kinase activity during HMG-CoA reductase assay is the availability of ATP-Mg in the medium. Addition of excess mevalonic acid (10 mM) before HMG-CoA reductase assay prevented the loss of [^{14}C]-mevalonate due to phosphorylated by mevalonate kinase.

Cytosol containing reductase kinase activity was able to inactivate (phosphorylate) purified HMG-CoA reductase, provided excess EDTA or mevalonic acid was added in the incubation. This inactivation was completely reversed by phosphatase. The 0-25% ammonium sulfate pellet (4 x washed) or DEAE sephacel fraction containing no mevalonate kinase were able to inactivate HMG-CoA reductase equally either in the presence or absence of EDTA and mevalonate. Incubation of inactivated enzyme with phosphatase was associated with complete restoration of enzymic activity. Fraction from DEAE sephacel column containing only mevalonate kinase activity failed to inactivate (phosphorylate) purified HMG-CoA reductase, therefore no dephosphorylation (reactivation) was observed following incubation with phosphatase. The inactivation of HMG-CoA reductase was time, ATP, and reductase kinase concentration dependent. In addition, the inhibition was not reversed by dilution or by addition of EDTA.

The combined results demonstrate unequivocally that mevalonate kinase and reductase kinase are two separate enzymes and furthermore that reversible inactivation (phosphorylation) of HMG-CoA reductase is catalyzed by reductase kinase. The artifactual effect of mevalonate kinase was totally eliminated either by using preparations free from mevalonate kinase or by the inclusion of excess EDTA or mevalonate before the assay of HMG-CoA reductase.

Significance to Biomedical Research and the Program of the Institute: The combined results represent the initial demonstration of the existence of active and inactive species of isolated rat liver HMG-CoA reductase. The interconversion of active and inactive species under different physiological situations may represent an additional mechanism (independent of phosphorylation-dephosphorylation) for the in vivo regulation of cellular cholesterol biosynthesis. The elucidation of the modes of control of this enzyme will allow a detailed analysis of the parameters involved in the cellular regulation of cholesterol metabolism in normal subjects and patients with atherosclerosis.

Proposed Course:

A systematic investigation of the characterization and modulation of active and inactive forms of HMG-CoA reductase will be continued. Modulation of HMG-CoA reductase will also be pursued in skin fibroblasts and lymphoblastoid cell lines from normal and familial hypercholesterolemia subjects.

Publications:

1) Beg, Z.H. and Brewer, H.B., Jr.: Regulation of liver 3-hydroxy-3-methylglutaryl coenzyme A reductase. Curr. Topics Cell. Reg. 20, 139-184, 1981.

2) Beg, Z.H., Stonik, J.A., and Brewer, H.B., Jr.: Isolation and immunological characterization of 3-hydroxy-3-methylglutaryl coenzyme A reductase from human liver. Biochem. Biophys. Res. Commun. 1982 (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02017-04 MDB

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Theoretical Analysis of the Metabolism of Lipoproteins and Their Apolipoprotein and Triglyceride Moieties

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Loren A. Zech, M.D.	Senior Investigator	MDB	NHLBI
Other:	Ernst J. Schaefer, M.D.	Senior Investigator	MDB	NHLBI
	H. Bryan Brewer, Jr., M.D.	Chief	MDB	NHLBI

COOPERATING UNITS (if any) Mones Berman, Ph.D., Laboratory of Theoretical Biology, NCI; S.M. Grundy, M.D., Ph.D., U. of Cal., La Jolla, CA; B.V. Howard, Ph.D., P.H. Bennett, M.D., Phoenix Clinical Research, Center Station, NIAMDI, W. Fisher, M.D., Ph.D., U. of Florida, Gainesville, Fl. and J.J. Albers, Ph.D., Seattle, WA.

LAB/BRANCH
Molecular Disease Branch

SECTION
Peptide Chemistry

INSTITUTE AND LOCATION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

ApoA-I synthesis is very sensitive to changes in the distribution of apoA-I among the slow and fast plasma components identified using the compartmental model to describe apoA-I metabolism in normal individuals. In Tangier disease metabolism from the fast plasma component for both homologous and autologous radiolabeled apoA-I is increased resulting in a decreased residence for this apoprotein.

Compared to the correlation between free fatty acid levels, VLDL-TG synthesis rates in Pima Indians, diabetic Pima Indians do not have this correlation with their continuously elevated free fatty acids but have a positive correlation between C-peptide (insulin) levels and VLDL-TG synthesis rate as determined using compartmental models.

Subjects with combined familial hyperlipoproteinemia have increases in both cholesterol and triglyceride synthesis compared to subjects with type IV hyperlipidemia with normal cholesterol and triglyceride synthesis rates but decreased VLDL-TG catabolic rate.

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Project Description:

1) Objective:

Development of both compartmental and statistical models for analysis of apoA-I and apoA-II metabolism in normal subjects and patients with disorders of apolipoprotein metabolism, lipoprotein metabolism, atherosclerosis, and other lipid abnormalities.

Methods Employed:

The methods used for the development of multicompartmental models using turnover data from radiolabeled apolipoproteins and plasma lipoprotein studies have been detailed in previous reports. These models are stimulated using the SAAM simulator (a large collection of digital computer programs on the Univac 1108 computer and the National Bureau of Standards, the Peripheral Data Processor 10 at the National Institutes of Health, and the VAX/780 digital computer in the Laboratory of Theoretical Biology, National Cancer Institute). These simulated results are compared to the experimental results and the connectivity (number and topology of compartments) as well as flow of the model changed until a working model is developed. Using this model, the volume of distribution of the apolipoproteins is estimated and compared to independent estimates of these volumes. After development of the compartmental model, the parameters of the model are adjusted using nonlinear least square techniques resulting in a minimal least square error. These have now been extended to studies in which differences in multicompartmental models, developed using turnover data from two separate radiolabels on two different apolipoproteins in normal individuals, have been compared.

Major Findings:

1) We continued to use sensitivity techniques to determine that synthesis (production) rates of apoA-I are very sensitive to changes in distribution of apoA-I between its two plasma components. The synthesis rate of plasma apoA-II is very insensitive to the separation of metabolism into two pathways.

2) In patients with Tangier disease apoA-I metabolism results in an increase of both the size and the catabolic rate of the fast metabolic component when compared to normals. This finding is consistent for both normal apoA-I, and apoA-I isolated from Tangier subjects.

2) Objective:

An analysis of the VLDL triglyceride kinetics of the Pima Indians.

Methods Employed:

The Pima Indian nation of Native Americans are an interesting, well-defined group of subjects. Studies of this group were performed in collaboration with Dr. Barbara Howard and Dr. Peter Bennett (Phoenix Experimental Station, NIAMDD). These individuals are of interest because they have an increased incidence of

diabetes, decreased incidence of obesity, and greater than 150% of ideal body weight. In addition, they also represent a well-defined group in which insulin, glucose, cholesterol, and LDL triglyceride kinetics have been previously studied.

VLDL-triglyceride was endogenously labeled following the injection of 300μCi of the radiolabeled precursor, ^{3}H-glycerol. In addition, plasma levels of insulin, glucose and free fatty acids were determined by standard techniques detailed in previous annual reports.

The kinetics of VLDL-triglyceride metabolism in normal triglyceridemia, non-diabetic, male, Pima Indians has been established and the relationships among the kinetic parameters (fractional catabolic rate, synthesis rate, delipidation rate, etc.) and plasma values of glucose, insulin and free fatty acids have been examined in detail. A group of 10 age matched male normal triglyceridemic diabetics have been examined using the compartmental model.

Major Findings:

1) The connectivity (number of compartments, and topology of tracer flow) of the model developed for the diabetic Pima Indians is equivalent to the model previously developed for both non-diabetic Pima Indians and caucasian males.

2) In contrast to the non-diabetic Pima Indians the synthesis rate of VLDL-TG does not correlate with plasma free fatty acid levels or with changes in free fatty acid levels.

3) The VLDL-TG production rate was strongly and positively correlated with the measurement of C-peptide values suggesting that insulin plays a major and more direct role in the short term modulation of TG synthesis in diabetics.

3) Objective:

1) Continued development of a multicompartmental model for triglyceride metabolism and the precursors of plasma lipoprotein triglycerides.

2) Estimation of the parameters describing triglyceride metabolism including synthesis rate, residence time, and fractional catabolic rate in groups of subjects with genetically determined hyperlipidemia.

Methods Employed:

1) Cohorts of subjects were selected by pedigree analysis and designated as having familial combined hyperlipidemia, or non-monogenic hypertriglyceridemia.

2) Radiolabeled glycerol was administered to patients in each of the two groups. The topological structure (connectivity and numbers of compartments) of multicompartmental models previously developed for triglyceride metabolism were examined for their ability to explain the experimental results in each group. After it was established that the current model could explain the observed kinetic data, the kinetic parameters of VLDL-triglyceride metabolism were calculated for each group.

Major Findings:

Analysis of kinetics of VLDL triglyceride kinetics was performed in collaboration with Dr. Mones Berman (Laboratory of Theoretical Biology, NCI), and Dr. Scott Grundy (Veterans Administration Hospital, La Jolla, CA.) In non-monogenic patients with hypertriglyceridemia the transport rates of VLDL-triglyceride (mg/hr) were not increased and the hypertriglyceridemia was secondary to a decreased fractional catabolic rate (FCR). In the familial combined hyperlipidemic group hypertriglyceridemia was associated with increased transport (production) rate however, a small subgroup of subjects also had a decreased fractional catabolic rate. While synthesis rates for cholesterol and bile acids were variable in patients with familial combined hyperlipidemia (as measured by balance techniques), overproduction of sterols is characteristic of this disorder. In contrast, most subjects in the non-monogenic hypertriglyceridemia group did not have increased sterol synthesis. A large enough number of subjects have now been examined such that these feelings have reached statistical significance.

4) Objective:

Extend the study of triglyceride kinetics to subjects with type V hyperlipoproteinemia. Also, to examine cholesterol metabolism using the cholesterol balance methods in type V subjects.

Methods Employed:

Subjects with type V hyperlipidemia were admitted to the metabolic ward where they were given a liquid formula diet for 28 days, low in fat and of sufficient calories to maintain weight, and known amounts of two non-absorbed stool markers, β-sitosterol and chromic acid.

Seven sequential four day stool samples were collected and examined for cholesterol, bile acids, and stool markers. These measurements were used to quantitate cholesterol and bile acid excretion and synthesis. In addition VLDL-triglyceride metabolism was examined following the injection of 300μCi of radiolabeled precursor, ^{3}H-glycerol, using previously developed compartmental models.

Major Results:

1) The experimental procedure has been performed in four type V subjects, and the patients were shown to have been in a steady state.

2) Based on the analysis of data from the first subject both cholesterol and triglyceride production rates were increased, suggesting that there is synthesis of both lipid components in type V subjects.

5) Objective:

Identification and characterization of subjects with a deficiency of LCAT (lecithin cholesterol acyltransferase) deficiency.

Major Findings:

One subject with an LCAT deficiency has been identified. This subject has 5% or normal LCAT mass and activity. This subject has an HDL or 2 to 6 mg/dl, a plasma triglyceride of 500 to 2000 mg/dl. This subject has plasma apoprotein levels of A-I= 27.1; A-II= 5.7; A-IV= 10.2; B= 42.7; C-II= 11.6; D= 4; E= 41.2; and H= 16.8; (mg/dl).

Significance to Biomedical Research and the Program of the Institute:

Understanding the metabolism of lipoproteins and the moieties which make up these lipoproteins (cholesterol, cholesterol ester, triglycerides, and apolipoproteins) are significant because of their relationship to atherosclerosis and cholelithiasis. The development of a concept of plasma lipoprotein metabolism is approached by the theoretical analysis of data from metabolic studies using the techniques of compartmental and statistical model building. This type of theoretical analysis provides a framework for discussion between investigators. This project consists of the testing and further development of compartmental models for lipoprotein metabolism as well as the proposal of new models where they do not exist.

Understanding lipoprotein metabolism is of major importance due to the central role of lipoproteins in the transport and catabolism of cholesterol and triglycerides in normal and patients with disorders of lipid metabolism and/or atherosclerosis. Becuase of the recent elucidation of the negative correlations between HDL-cholesterol levels and the incidence of coronary heart disease the understanding of the two major HDL apolipoproteins (apoA-I and apoA-II) is particularly relevant to the understanding of atherosclerosis.

Understanding the modulation of lipoprotein, cholesterol, cholesterol ester, triglycerides and apolipoproteins by drugs, diet, and genetic disease is also of significance since changes in these effectors may have major effects on atherosclerosis and cholelithasis. This theoretical analysis also provides a framework for comparison between groups as dissimilar as caucasians and American Indians.

Proposed Course:

Detailed studies will be continued on the analysis of the differences in apoA-I and apoA-II metabolism by further specifying and defining the current compartmental models, with particular emphasis on analysis of studies in abnormal subjects such as type I and Tangier subjects. Additional Pima Indians will be examined before and after weight loww to see if control moves from free fatty acid levels to c-peptide (insulin) levels. Preliminary studies will be continued on the compartmental analysis of apoE and apoC metabolism. The overall objective will be the development of a comprehensive model of human lipoprotein metabolism by the incorporation of this formation into previously proposed LpB models. The formulation of an overall conceptualization of lipoprotein metabolism will be continued by qualitative and quantitative testing of these conceptions using compartmental modeling and other theoretical methods. Of particular interest will also be the determination of which parameters are modified by diet, drug, and transformed by genetic disease.

In addition, laboratory examination of type V subjects using the cholesterol balance method and the triglyceride, radiolabeled glycerol method will be carried out on 3 to 6 more subjects.

Also, the concepts of sensitivity analysis applied to compartmental analysis will be further developed and expanded to aid in detailed studies proposed above.

Publications:

1. Ghiselli, G., R.E. Gregg, L.A. Zech, E.J. Schaefer and H.B. Brewer, Jr.: (1982) Apolipoprotein E isoforms in patients with hyperlipoproteinemia: A phenotype study. Lancet (in press).

2. Schaefer, E.J., L. Kay, L.A. Zech and H.B. Brewer, Jr.: (1982) J. Clin. Res. Tangier Disease: High density lipoprotein deficiency due to defective metabolism of an abnormal apolipoprotein A-I (ApoA-$I_{Tangier}$). JCI (in press).

3. Howard, B.V., L.A. Zech, J.S. Reitman, M.P. Davis and S.M. Grundy: (1982) Studies of very low density lipoprotien triglyceride metabolism in Pima Indians. In Lipoprotein Kinetics and Modeling (ed. by M. Berman, S. Grundy, B. Howard). Academic Press, New York (in press).

4. Ghiselli, G., E.J. Schaefer, L.A. Zech, R.E. Gregg and H.B. Brewer, Jr.: (1982) Increased prevalence of apolipoprotein E_4 in type V hyperlipoproteinemia. J. Clin. Invest. (in press).

5. Schaefer, E.J., L.A. Zech, R.E. Gregg and H.B. Brewer, Jr.: (1982) Metabolism of high density lipoproteins. In Proceedings of the US-USSR Symposium on Lipoprotein Metabolism, NIH; 82-1966 (in press).

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7. Beil, U., S.M. Grundy, J.R. Crouse and L.A. Zech: (1982) Triglyceride and cholesterol metabolism in primary hypertriglyceridemia. Arteriosclerosis 2: 44-57.

8. Brewer, H.B., Jr., L.A. Zech, R.E. Gregg, D. Schwartz and E.J. Schaefer: (1982) Recent advances in the diagnosis, molecular defects, pathology, and treatment of type III hyperlipoproteinemia. Arch. Int. Med. (in press).

9. Brewer, H.B., Jr., E.J. Schaefer, J.C. Osborne, Jr. and L.A. Zech: Recent advances in the structure, function and metabolism of human plasma lipoproteins. In Lipoproteins and Aging, H. Kaffarmik, ed. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02019-04 MDB

PERIOD COVERED October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Metabolism of Human Plasma Apolipoproteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Richard E. Gregg, M.D.	Clinical Associate	MDB	NHLBI
Other:	Ernst J. Schaefer, M.D.	Senior Investigator	MDB	NHLBI
	Loren A. Zech, M.D.	Senior Investigator	MDB	NHLBI
	Giancarlo Ghiselli, Ph.D.	Visiting Fellow	MDB	NHLBI
	Rosemary Ronan, B.A.	Chemist	MDB	NHLBI
	Elizabeth A. Rubalcaba, B.A.	Chemist	MDB	NHLBI
	Leslie Jenkins, M.S.	Chemist	MDB	NHLBI
	Diana Wilson, M.S.	Chemist	MDB	NHLBI
	H. Bryan Brewer, Jr., M.D.	Chief	MDB	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH Molecular Disease Branch

SECTION Peptide Chemistry

INSTITUTE AND LOCATION NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 3.5	PROFESSIONAL: 1.5	OTHER: 2.0

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Apolipoprotein E (apoE) is important in the metabolism of lipoproteins. In humans there are 3 common forms of apoE; apoE-2, apoE-3, and apoE-4. ApoE levels, apoE phenotypes, and the metabolism of apoE forms were studied. Elevated apoE concentrations were found in all groups of hyperlipidemic subjects. Previous reports of an association of apoE-2 with type III hyperlipoproteinemia (HLP) were confirmed and, in addition, apoE-4 was clearly associated with type V HLP. A new variant of apoE, apoE-Bethesda, associated with type III HLP was also discovered. ApoE kinetic studies were performed in normal and type III HLP subjects. The elevated apoE levels in type III subjects were due to both decreased catabolism and increased production. In normal subjects apoE-4 had a shorter residence time than apoE-3, and this unexpected finding suggests that apoE has another function in addition to regulating lipoprotein remnant catabolism. These results indicate that both apoE-2 and apoE-4 are associated with hyperlipidemic states, that apoE-2 and apoE-4 are metabolically distinct from apoE-3, and that additional information about apoE metabolism may be gained by screening for apoE variants and studying the metabolism of apoE in unusual forms of dyslipidemia.

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PHS-6040
(Rev. 2-81)

Project Description

Objective

The purpose of this project was to study the relationship of apoE levels, phenotypes, and kinetics to primary and secondary HLP conditions. It has been proposed that in humans there are 3 common alleles expressed for apoE (E^2, E^3, and E^4) inherited at one genetic locus with codominant expression of these alleles. This results in 6 genotypes, 3 homozygous and 3 heterozygous, and these genotypes can be inferred from the apoE phenotypes determined by isoelectric focusing polyacrylamide gel electrophoresis. It has previously been shown that the E^2 allele is associated with type III hyperlipoproteinemia and these individuals have elevated apoE levels. There have also been reports of normal apoE phenotypes with slight to moderate increases in the plasma apoE concentration in a small group of hyperlipidemic subjects; but there has not been a comprehensive study of apoE phenotypes and levels in a large group of hyperlipidemic and dyslipidemic subjects. In addition, nothing is known about the kinetics of apoE metabolism in humans other than the previous studies reported from our laboratory on a small group of normal and type III subjects. Therefore, this project was designed to determine the apoE levels in subjects with the common HLP phenotypes and other rare dyslipidemic states and to simultaneously determine their apoE phenotypes by one-dimensional or two-dimensional gel electrophoresis. An additional purpose of this study was to determine the kinetic parameters of the metabolism of the different forms of apoE in normal subjects with primary or secondary hyperlipidemia. These studies should provide information about factors regulating the metabolism of apoE and lipoproteins and remnants of lipoproteins, and give insights into pathophysiologic mechanisms of hyperlipoproteinemic syndromes.

Methods Employed

Using a sensitive double antibody radioimmunoassay previously developed in our laboratory, the plasma apoE concentration was quantitated in a large number of normal, hyperlipoproteinemic and dyslipoproteinemic subjects. In addition, these subjects had their apoE phenotypes determined by one-dimensional isoelectric focusing polyacrylamide gel electrophoresis (IEF-PAGE) or two-dimensional gel electrophoresis (IEF-PAGE followed by SDS-PAGE).

$ApoE_2$, $apoE_3$, and $apoE_4$ were purified to homogeneity by ultracentrifugation, delipidation, heparin affinity chromatography, and gel filtration chromatography. The three purified forms of apoE were radioiodinated by the iodine monochloride method and injected into normal subjects and patients with either primary or secondary hyperlipidemia.

The rate of decay of the radiolabeled apoE from plasma and lipoprotein subfractions was quantitated by computer modelling methods and the residence times, fractional catabolic rates, and production rates of apoE were determined. In addition, the distribution of endogenous and radiolabeled apoE was determined in the lipoprotein subfractions separated both by ultracentrifugation and by gel permeation chromatography.

Major Findings

1) The mean plasma apoE concentration in normal lipidemic subjects was 5.7 mg/dl ± 1.4 (± SD). There was no significant association with sex or age.

2) All hyperlipoproteinemia phenotypes were associated with elevated apoE levels.

Hyperlipoproteinemia Phenotype		ApoE Concentration mg/dl
Type I	n = 11	28.8 ± 7.1 (± SD)
Type IIa	n = 16	7.9 ± 1.9
Type IIb	n = 11	11.8 ± 3.3
Type III	n = 10	38.6 ± 12.9
Type IV	n = 17	13.0 ± 5.3
Type V	n = 11	47.1 ± 21.6
Normal	n = 58	5.7 ± 1.4

3) ApoE levels were determined in a number of rare dyslipoproteinemic conditions. Elevated apoE levels were found in subjects with betasitosterolemia, abetalipoproteinemia, Wolman's disease, LCAT deficiency, and primary biliary cirrhosis, while the apoE levels were low in Tangier's disease and apoE deficiency.

4) The prevalence of the 3 alleles for apoE in our normal population was very similar to that reported by others previously. In our population the relative allelic frequency of E^2 was 9.5%, E^3 was 77.0% and E^4 was 13.5%.

5) As previously reported, we found a very high frequency of the E^2 allele in subjects with type III hyperlipoproteinemia; the E^2 allele being 87% of all the E alleles in these subjects.

6) We clearly documented a new association of a very high prevalence of the E^4 allele in subjects with type V hyperlipoproteinemia. They had an E^4 allelic frequency of 51.7% compared to 13.5% in normals.

7) The first variant of apoE, which we have designated $apoE_{Bethesda}$, was discovered in a subject with type III hyperlipoproteinemia. This individual had a heterozygous phenotype for apoE with two major isoforms on IEF-PAGE. One isoform migrated in the usual E_2 position but one isoform was

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found in the E_1 position; a position where a major isoform has not previously been found. On family screening one offspring was also found to have $apoE_{Bethesda}$, with one major form in the E_3 position and another in the E_1 position, and type III hyperlipoproteinemia. $ApoE_2$ is associated with type III hyperlipoproteinemia in a recessive genetic mode, i.e., most type III subjects are homozygous for the E^2 allele and, in marked contrast, $apoE_{Bethesda}$ was associated in a dominant mode. On chemical analysis, $apoE_{Bethesda}$ was demonstrated to contain 2 cysteine residues, as does $apoE_2$, and therefore it is likely that $apoE_{Bethesda}$ represents a mutation of the E^2 allele.

8) The metabolism of $apoE_2$ and normal apoE ($apoE_3$) was studied in type III and normal subjects homozygous for E^2 and E^3 respectively. Compared to $apoE_3$, $apoE_2$ was found to be catabolized significantly slower in both normal and type III subjects. It was found that $apoE_2$ in type III subjects had one-half the fractional catabolic rate of $apoE_3$ in normal subjects and, in addition, the synthesis rate of $apoE_2$ was five times greater. These data indicate that $apoE_2$ is catabolized abnormally and that the elevated $apoE_2$ levels in type III subjects is due to a dual defect. The increased plasma apoE level is due primarily to over-production of apoE with decreased fractional catabolism also playing a significant role.

9) A high prevalence of $apoE_4$ in type V hyperlipoproteinemic subjects was discovered during screening. The metabolism of $apoE_3$ and $apoE_4$ was therefore studied in normal individuals and one type V subject with an E_4 phenotype. Compared to radiolabelled $apoE_3$, $apoE_4$ was distributed more in VLDL and less in HDL both by column chromatography and ultracentrifugation. In addition, $apoE_4$ had twice the fractional catabolic rate of $apoE_3$. The distribution and kinetic findings of purified radiolabelled $apoE_4$ were supported by the level and distribution of endogeneous apoE in a normal lipidemic subject with an E_4 phenotype; his apoE level was less than one-half of normal and there was proportionately more apoE in his VLDL compared to other normal subjects with an E_3 phenotype. The elevated apoE level in the type V subject was predominantly due to an increased production rate. We conclude from these data that $apoE_4$ is metabolically distinct from $apoE_3$ and is probably important in the pathogenesis of type V hyperlipoproteinemia. The surprising finding of an enhanced fractional catabolic rate of $apoE_4$ suggests there is a second metabolic function for apoE in addition to facilitating the catabolism of lipoprotein remnants.

10) ApoE kinetic studies were performed in a group of patients with either rare or secondary forms of dyslipoproteinemia. Two patients with abetalipoproteinemia had elevated apoE levels which were entirely due to increased production rates. A subject with apoE deficiency had a significantly decreased fractional catabolic rate for apoE reflecting the increased levels of VLDL and IDL that were being slowly catabolized. The apoE level in a patient with LCAT deficiency was increased which was secondary to an increased production rate. Four subjects with primary biliary cirrhosis and elevated apoE levels were studied; one early in the disease, one in mid-disease, and two with end-stage disease. The early disease patient had an elevated HDL cholesterol level, a decreased fractional catabolic rate for apoE, and an increased production rate; the mid-disease patient had a very high HDL cholesterol level, the highest apoE level measured in our laboratory, a

slightly decreased fractional catabolic rate, and a very high synthesis rate; the two end-stage disease patients had very low HDL cholesterol levels, an increased fractional catabolic rate for apoE and a markedly increased production rate. There was an elevated plasma concentration and increased synthesis rate for apoE in all of these disease states. The fractional catabolic rate was variable, being decreased when there was either an increased HDL cholesterol level or an increased amount of remnants of triglyceride-rich lipoproteins that were being slowly catabolized.

Significance to Biomedical Research and the Program of the Institute

ApoE is an important modulator of the metabolism of lipoproteins. Our studies have indicated that $apoE_2$ is associated with type III HLP and $apoE_4$ with type V HLP. These associations are of clinical significance because they are likely to give insights into the pathophysiology of type III HLP, which predisposes to xanthomatosis and premature cardiovascular and peripheral vascular disease, and type V HLP, which predisposes to eruptive xanthomatosis, pancreatitis, arthralgias, myalgias and peripheral neuropathies. We have demonstrated that both $apoE_2$ and $apoE_4$ are metabolically abnormal compared to $apoE_3$. We have also shown that primary and secondary hyperlipidemic states have both synthetic and catabolic abnormalities leading to elevated apoE levels. These findings, along with the discovery of a variant of apoE, $apoE_{Bethesda}$, that is associated with type III HLP, have given new insights into the function of apoE in lipoprotein metabolism and the control of the metabolism of apoE itself.

We have confirmed and extended previous findings that apoE is important in the catabolism of lipoprotein remnant particles and that the elevated apoE and lipoprotein remnant levels in type III HLP are due both to oversynthesis and a decreased fractional catabolic rate. The findings of an increased prevalence of $apoE_4$ in type V HLP subjects and its abnormal metabolism make it likely that the apoE is important in the modulation of the metabolism of chylomicrons and VLDL. The studies of apoE metabolism in secondary forms of hyperlipidemia demonstrate that not only does the form of apoE present in plasma modulate lipoprotein metabolism but the type of lipoprotein particle present modulates the metabolism of apoE. By continuing these studies more knowledge will be gained into the regulation of lipoprotein metabolism by apoE, the metabolic defects in certain forms of hyperlipoproteinemia will be understood at a molecular level, and it will be possible to treat these hyperlipoproteinemic states and their secondary complications on a more complete and rational basis.

Proposed Course

We propose to continue studying apoE at a structural, functional, and kinetic level. A screening program will continue to identify rare variants of apoE associated with dyslipidemic states, and the known and new forms of apoE will be studied by detailed chemical and physical analysis. The function of apoE will be studied using apoE binding studies to apoE receptors on hepato-

cytes and by evaluating the modulating affect of apoE on the lipolytic system. New variants of apoE, such as $apoE_{Bethesda}$, will be studied kinetically to determine the functional significance of the structural abnormality. Similarly, apoE with modified cysteine residues will be studied kinetically to determine the importance of these residues in the function and metabolism of apoE. $ApoE_4$ kinetics will be studied in additional type V subjects to measure their apoE synthesis and catabolic rates. And finally, the metabolism of apoE will be studied in type III HLP subjects before and after estrogen therapy to determine its affect on apoE kinetics.

Publications

1. Schaefer, E. J., Zech, L. A., Gregg, R. E., and Brewer, H. B., Jr.: Metabolism of high density lipoproteins. Proceedings of the US-USSR Symposium of Lipoprotein Metabolism (in press).

2. Ghiselli, G., Schaefer, E. J., Zech, L. A., Gregg, R. E., and Brewer, H. B., Jr.: Increased prevalence of apolipoprotein E_4 in type V hyperlipoproteinemia. J. Clin. Invest. (in press).

3. Brewer, H. B., Jr., Zech, L. A., Gregg, R. E., Schwartz, D., and Schaefer, E. J.: Recent advances in the diagnosis, molecular defects, pathology, and treatment of type III hyperlipoproteinemia. Ann. Int. Med. (in press).

4. Ghiselli, G., Gregg, R. E., Zech, L. A., Schaefer, E. J., and Brewer, H. B., Jr.: Apolipoprotein E isoforms in patients with hyperlipoproteinemia: a phenotype study. Lancet (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02021-02 MDB

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Sterol Metabolism in Patients with Betasitosterolemia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Richard E. Gregg, M.D.	Clinical Associate	MDB	NHLBI
Other:	Jeffrey M. Hoeg, M.D.	Research Associate	MDB	NHLBI
	H. Bryan Brewer, Jr., M.D.	Chief	MDB	NHLBI

COOPERATING UNITS (if any)
William Connor, M.D., Dept. of Medicine, University of Oregon Health Center Portland, Oregon; George Vahouny, Ph.D., and Linda Gallo, Ph.D., Dept. of Biochemistry, George Washington University, Washington, D.C.

LAB/BRANCH
Molecular Disease Branch

SECTION
Peptide Chemistry

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Subjects with betasitosterolemia are characterized clinically by having xanthomas in childhood and premature cardiovascular disease. Biochemically these patients are distinguished by having elevated plasma levels of all dietary sterols, particularly the plant sterol betasitosterol. We have previously shown that in this disease there is an overabsorbtion of cholesterol, plant sterols, and shellfish sterols. We have now shown that there also is an abnormal ability to concentrate sitosterol and shellfish sterols, relative to cholesterol, in the bile. In addition we have shown that even though other dietary sterols are present in xanthomas, cholesterol is still the predominant sterol indicating that cholesterol is not metabolized normally in this tissue. These data suggest that betasitosterolemic subjects have a pansterol metabolic abnormality and that this defect is present in all tissues of the body.

784

Project Description:

Objectives:

Subjects with betasitosterolemia have been shown to overabsorb cholesterol, plant sterols, and shellfish sterols. The purpose of this project was to determine if there was evidence for abnormal sterol metabolism in other tissues of the body and to define the abnormality in betasitosterolemia at a molecular level.

Methods Employed:

A patient with betasitosterolemia was placed on a diet high in shellfish. Sterol absorption was determined by the sterol balance method, bile was obtained via a nasogastric tube and analyzed, and the plasma sterols were characterized and quantitated. In addition the sterols from a xanthoma from this patient were quantitated. A small bowel biopsy was obtained to determine if there were any morphologic abnormalities at the light or electron microscopic level.

A skin biopsy was obtained from this subject to culture fibroblasts to determine the metabolism of sterols at a cellular and molecular level. Radiolabeled cholesterol, sitosterol, and oleyl-CoA were used to evaluate the ability of these cells to take up, esterify, deesterify, and excrete cholesterol and sitosterol. In addition pancreatic secretious were obtained from the duodenum and evaluated for the specificity of the pancreatic steryl ester hydrolase activity using cholesteryl and sitosteryl esters as substrates.

Major Findings:

1) As was found previously, our subject with betasitosterolemia had increased absorbtion of shellfish sterols and elevated plasma levels of these sterols.

2) Relative to cholesterol, our subject had a marked decrease in the concentration of sitosterol and shellfish sterols in bile compared to normal individuals.

3) The predominant sterol in xanthomas from the subject was cholesterol, even though other dietary sterols were present. The relative concentrations of the xanthoma sterols reflected the relative concentrations of the plasma sterols.

4) A small bowel biopsy showed increased lipid within the intestinal mucosal cells but otherwise normal structure of the tissue.

5) The pancreatic steryl ester hydrolase deesterified cholesterol and sitosterol normally.

6) Preliminary studies on this subject's fibroblasts did not reveal any abnormalities in these cells but more definitive studies have not been completed to fully evaluate the sterol metabolism.

Significance to Biomedical Research and the Program of the Institute:

Betasitosterolemia is characterized by abnormalities in sterol metabolism in a number of different tissues. There is overabsorption of dietary sterols, increased deposition of sterols in tissues with only mildly elevated total plasma sterol levels, and decreased excretion of sterols in bile. These are associated clinically with xanthoma formation in childhood and premature cardiovascular disease. We are seeking to elucidate the abnormality in this disease at a molecular level in order to improve our understanding of the pathophysiology of betasitosterolemia. Of even broader importance is the insight that the understanding of this disease will give to the more general problems of the cellular control of sterol absorption and excretion, the regulation of xanthoma formation, and the role of abnormal intracellular sterol metabolism in the atherosclerotic process.

Proposed Course:

It is planned to do additional sterol balance studies on betasitosterolemic subjects while on different therapeutic diet and drug regimens. In addition, the bile will be more extensively evaluated than has been done previously during these studies and intracellular sterols and steryl esters will be quantitated. The cell culture studies will continue and it is planned to determine if there is altered specificity of intracellular sterol binding proteins and the enzymes involved in sterol metabolism for cholesterol and sitosterol. These studies will be conducted in fibroblasts and if technically feasible, in intestinal mucosal cells from normal and betasitosterolemic subjects.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02022-02 MDB

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Cellular Lipid and Lipoprotein Biochemistry and Metabolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Jeffrey M. Hoeg, M.D.	Research Associate	MDB	NHLBI
Others:	H. Bryan Brewer, Jr., M.D.	Chief	MDB	NHLBI
	Stephen Demosky, Jr.	Chemist	MDB	NHLBI
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COOPERATING UNITS (if any)

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LAB/BRANCH
Molecular Disease Branch

SECTION
Peptide Chemistry

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.2	1.2	2.0

CHECK APPROPRIATE BOX(ES)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Research in our laboratory is directed toward the characterization and quantitation of lipoprotein-cell interactions and its impact on cellular cholesterol and cholesteryl ester metabolism. We have recently developed methods for quantitating and analyzing freely reversible lipoprotein binding to intact cells as well as isolated membranes from several human sources. Using these techniques, insights have been made on the role of the low density lipoprotein receptor on cholesterol transport and its importance in modulating intracellular enzymes crucial to cholesterol and cholesteryl ester metabolism. 3-hydroxy-3-methylglutaryl coenzyme A reductase, acid ester hydrolase and neutral ester hydrolase studies conducted on tissues from patients homozygous for familial hypercholesterolemia suggest that the receptor defect for low density lipoproteins results in enhanced hepatic biosynthesis of low density lipoproteins. Previous studies on intracellular cholesterol metabolism in Wolman's disease and Cholesteryl Ester Storage Disease have been extended and the studies on niacin and neomycin as hypocholesterolemic agents in patients with type II lipoprotein phenotypes and the value of niacin in lipoprotein phenotypes III and V continue.

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Project Description

Objective

1) Evaluation of lipoprotein-cell interaction *in vitro* with intact human cell lines

By studying the interaction of different lipoproteins *in vitro* with different intact human cell lines insights on normal lipoprotein lipid transport function as well as understanding the integration of lipoprotein receptor function with intracellular lipid metabolism can be derived.

Methods

Human skin fibroblasts were grown on a microcarrier bead system as outlined in last year's annual report. Using this system, the adherent fibroblasts grow in the media as a cell suspension. One advantage of this cell suspension system is that the fibroblasts can be rapidly separated from the media by sedimentation, centrifugation or filtration, thus allowing for the first time the direct evaluation of freely reversible binding of radiolabeled ligands to adherent cell lines. A second advantage to the cell suspension system is that adherent cell lines can be exposed to constant media conditions through the use of a continuous perfusion system. This obviates swings in media composition which could affect the results of a variety of biochemical studies. The ability of these fibroblasts to reversibly bind, irreversibly internalize, and degrade iodinated low density lipoproteins (^{125}I-LDL) was assessed in the assay systems we developed last year.

One limitation in the full *in vitro* characterization of the effects of lipoproteins on cellular lipid metabolism in human cells is the small amount of material available for study. The purification and characterization of human receptors and enzymes and study of their modulation by the presence of different lipoproteins require more tissue than is available using current techniques. One method we evaluated for obviating this limitation has been to transform human lymphocytes to a malignant cell line by infection with Epstein-Barr virus. Lymphocytes from normal subjects and patients homozygous for familial hypercholesterolemia were transformed and grown in large volume. The ability of these cells to bind ^{125}I-LDL as well as to regulate the rate limiting step of cholesterol biosynthesis 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) was then measured using the standard assay procedures of Beg *et al*.

In all these studies the preparation, characterization, and radioiodination of plasma lipoproteins were performed using standard techniques of preparative ultracentrifugation, triglyceride, cholesterol, protein quantitation and iodine monochloride iodination.

Finally, using mouse peritoneal macrophages and human macrophages, the role of different lipoproteins from normal and dyslipidemic patients in lipid transport to macrophages was evaluated. Using new enzymatic, fluorometric assays,

we were able to quantitate picomolar quantities of cholesterol, cholesteryl ester and triglyceride in tissue culture preparations.

Major Findings

1) Using the microcarrier-bead system, we demonstrated for the first time that freely reversible LDL-fibroblast binding is not only present in fibroblasts from normal subjects, but also that this important first step in lipoprotein-cell interaction accounts for greater than half of the ^{125}I-LDL adsorbed to the cell surface.

2) The microcarrier-bead fibroblast cultures can be grown in suspension for long periods (3 weeks or longer) and can be adapted to continuous perfusion culture techniques.

3) Lymphocytes transformed by Epstein-Barr virus from normal humans demonstrate the same regulation of HMG-CoA reductase and LDL receptor concentration observed in fibroblast cell lines. Furthermore, transformed lymphocytes from patients homozygous for familial hypercholesterolemia conserve the LDL recognition defect observed in their fibroblasts.

4) Serum, plasma and lipoprotein subfractions from patients from type I, III, IV and V induce massive macrophage lipid accumulation in both mouse peritoneal and human derived macrophages. Serum, plasma, and lipoproteins from normal subjects do not induce this accumulation.

Objective

2) Characterization and quantitation of hepatic membrane binding of lipoproteins

The liver plays a central role in mammalian cholesterol metabolism. It not only synthesizes most of the body's cholesterol but it also is the main site of cholesterol excretion from the body in the form of bile acids. The first step in the removal of lipoproteins from the plasma is through the binding of the lipoproteins to the lipoprotein receptors within the plasma membrane. Although hepatic lipoprotein receptors have been characterized in several mammalian species, the presence of these receptors and their physiologic role in normal humans as well as their pathophysiologic role in dyslipidemic patients has not been previously evaluated. Thus we set out to develop assay techniques for the LDL receptor in hepatic membranes and to measure the hepatic LDL receptor number and function in normal subjects and familial hypercholesterolemic patients.

Methods

The techniques for hepatic membrane isolation were developed using rat liver preparations and the assay buffer, and incubation temperature and time were optimized for evaluation of ^{125}I-LDL binding and then applied to isolated human liver membranes. In collaboration with Dr. Thomas Starzl at the University of Pittsburgh we obtained hepatic tissue at the time of therapeutic

portocaval shunt of two homozygotes with LDL receptor negative familial hypercholesterolemia. The binding of ^{125}I-LDL binding was directly compared to that in normal human liver.

Major Findings

1) Previously reported binding assays underestimated the degree of specific ^{125}I-LDL binding to normal rat liver membranes because of the incubation conditions used. Significantly more binding of ^{125}I-LDL occurs when membranes are incubated at 37°C than at 0°C. Furthermore, at least half of the specific ^{125}I-LDL binding to rat liver membranes is not calcium dependent and has therefore been overlooked by previous investigators.

2) The LDL receptor in the rat appears to be coordinately regulated with maneuvers which alter the degree of HMG-CoA reductase activity such as feeding and cholestyramine treatment.

3) Normal adult human liver demonstrates a considerable degree of recognition for LDL. This suggests that the human liver may play an important role in the removal of LDL from the plasma.

4) Patients lacking the LDL receptor in non-hepatic tissues, familial hypercholesterolic homozygotes, also have defective hepatic binding to LDL. FH homozygotes produce 2-4 fold more LDL than normals, the defective recognition of LDL by the liver may be the proximate cause of the striking hypercholesterolemia and ultimately of the accelerated premature atherosclerosis experienced by these individuals.

Objective

3) Evaluation of Inborn Errors of Cholesteryl Ester Metabolism

By studying tissue from subjects with an isolated enzymic defect, the normal role of the enzyme as well as its relationship to other cellular processes are defined. Acid ester hydrolase is a lysosomal enzyme used to hydrolyze ester bonds in cholesteryl esters as well as triacylglycerols. Patients with Wolman's Disease and Cholesteryl Ester Storage Disease both are deficient in the activity of this enzyme. By studying the degree of the loss of this enzyme as well as differences in the metabolic consequences of the loss, differences in clinical course as well as insights to more effective therapy might be found.

Methods

Assays for neutral acid ester hydrolase were developed as outlined in last year's annual report. Assays were performed on skin fibroblast cultures and liver biopsy specimens from patients with Wolman's Disease and Cholesteryl Ester Storage Disease. Cholesterol, cholesteryl ester and HMG-CoA reductase activities were also quantitated.

Major Findings

Despite the loss of acid ester hydrolase activity in the tissues from these subjects, the neutral ester hydrolase activity remained normal. This suggests that these two enzymes are genetically distinct.

Objective

4) Treatment of type II hyperlipoproteinemic subjects with neomycin and niacin

Patients with type II hyperlipoproteinemia have elevated plasma cholesterol levels and premature atherosclerosis. Treatment is successful in lowering plasma cholesterol levels in less than half of the patients because the current first-line drug, cholestyramine, is unpalatable. The antibiotic neomycin in uncontrolled trials has a marked hypocholesterolemic effect. The goal of this outpatient study of neomycin is to evaluate both its efficacy and its safety.

Methods

This is a double-blind, placebo controlled, randomized, cross-over trial of neomycin and the combination of neomycin and niacin. Presently, 28 subjects are enrolled in this outpatient study and come to clinic for monthly lipoprotein, renal, hepatic, nutritional and audiologic assessment.

Major Findings

The study is in progress and the evaluation of the first cross-over period is now being analyzed. To date, no serious side-effects of neomycin treatment has been noted.

Significance to Biomedical Research and the Program of the Institute

By evaluating the interaction of lipoproteins with intact human cell lines and isolated subcellular fractions from different tissues in vitro, insights on the normal physiologic lipid transport function of the lipoproteins and their coordination with intracellular lipid metabolism can be derived. In addition, these studies can be extended to evaluate possible pathophysiologic mechanisms of the dyslipidemias. Information on specific molecular defects in the dyslipidemias and the metabolic consequences of these defects is necessary for an understanding of these disease processes and may ultimately lead to more effective treatment for these disorders.

Proposed Course

Now that the fibroblast-bead carrier system and the transformed lymphoctye technique has been characterized, these systems will be used to evaluate the integration of lipoprotein receptor modulation with intracellular lipid composition and enzymes central to cellular cholesterol metabolism. Evaluation of macrophage foam cell production and the characterization of the lipoproteins responsible for this phenomenon will be extended. The interaction of normal human hepatic membranes with lipoproteins other than LDL

will be assessed and the coordination of lipoprotein uptake, synthesis, and lipoprotein receptors will be studied in pertinent animal models of human dyslipidemias. Finally, the outpatient clinical trial of neomycin and niacin will be continued.

Publications:

1. Hoeg, J. M., Demosky, S. J., Brewer, H. B., Jr.: Characterization of neutral and acid ester hydrolase in Wolman's disease. Biochim. Biophys. Acta 711: 59-65, 1982.

2. Hoeg, J. M., Osborne, J. C., Jr., Brewer, H. B., Jr.: Analysis of freely reversible lipoprotein-cell interactions. J. Biol. Chem. 257 (5): 2125-2128, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02024-01 MDB

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Molecular Biology of Plasma Apolipoproteins and Lipoproteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Simon Law	Expert	MDB	NHLBI
Other:	H. Bryan Brewer, Jr.	Chief	MDB	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Molecular Disease Branch

SECTION
Peptide Chemistry

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A library of cloned genes for plasma proteins has been prepared from human liver. mRNA was fractionated by guanidinium thiocyanate extraction, and purified by affinity chromatography. cDNA was prepared using reverse transcriptase, and the cDNA was cloned in E-coli plasmid pBR322. Screening for specific genes has been initiated using synthetic polynucleotides prepared from the known amino acid sequences of plasma proteins and apolipoproteins. Several of the clones have been shown to contain the gene coding for human albumin. Current studies are underway to identify the clones containing genes which code for apolipoproteins A-I and E.

793

PHS-6040
(Rev. 2-81)

Project Description:

Objective:

1) Development of library of cloned genes for plasma proteins from human liver.

Methods Employed:

Human liver tissue was obtained during surgery. mRNA was isolated by guanidinium thiocyanate extraction procedure followed by affinity column chromatography. cDNA was then synthesized on the mRNA template using the enzyme reverse transcriptase and cloned into the Pst-I restriction endonuclease site of E-coli plasmid pBR322. Screening for specific clones has been initiated by the preparation of synthetic polynucleotides coding for the known amino acids sequence of apolipoproteins.

Major Findings:

cDNA clones have been obtained by transforming E-coli RR1 with recombinant plasmid DNA followed by drug selection. The number of clones are high enough to cover mRNA sequences representing 0.01% of total liver polyadenylated RNA. Screening was performed by colony hybridization to radiolabelled cDNA synthesized by prime extension of mRNA with polynucleotides specific for the apolipoproteins. Clones that are complementary to the radiolabelled cDNA are selected and identified by Maxim and Gilbert DNA sequencing and hybrid selection. Currently identified clones containing the nucleic acid sequence of human serum albumin and an unknown protein have been identified. Specific hybridization of polynucleotide prime synthesized cDNA to clones containing long sequences of human serum albumin clones but not clones with partial inserts and as yet an unidentified protein that is encoded by mRNA representing only 0.5 to 1% of total liver mRNA population have demonstrated the specificity of the polynucleotide priming reaction.

Objective:

2) Screening of clones which contain the genes coding for plasma apolipoproteins.

Methods Employed and Major Findings:

The library of cloned genes from human liver prepared as outlined above will be used to obtain the genes coding for plasma apolipoproteins.

The screening for specific clones has been initiated by the preparation of synthetic polynucleotides prepared from the known sequences of the apolipoprotein. Studies are currently underway to determine the clones which contain the genes for apolipoproteins apoA-I and E.

Significance to Biomedical Research and Program of the Institute: An understanding of the molecular organization of the genes coding for the plasma apolipoproteins will be of major interest and importance in our ultimate understanding of the structure, function, and regulation of the plasma apolipoproteins. Of particular interest will be information related to the organization of the human genes for apolipoprotein with respect to the coding of signal peptides for apolipoproteins, and if introns are present in the genes. Information on the genes coding for normal apolipoproteins will permit a detailed analysis of the genetic apolipoprotein abnormalities found in patients with dyslipoproteinemia.

Proposed Course:

Studies will be continued on the identification of the clones containing sequences which code for the human plasma apolipoproteins. Major emphasis will be placed on the cloning of the genes for apolipoproteins E and A-I. Cell free translation studies are under development and will be used in the analysis of the signal peptides associated with human apoA-I and apoE. Of particular interest will be the analysis of the genetic defect in Tangier disease which has recently been shown to be a structural defect in apoA-$I_{Tangier}$ and an analysis of the newly discovered patients who have apoE deficiency.

Publications:

Achilles Dugaiczyk, Simon W. Law and Olivia E. Dennison: Nucleotide sequence and the encoded amino acids of human serum albumin mRNA. Proc. Natl. Acad. Sci. USA. 79: 71-75, 1982.

Simon W. Law and Achilles Dugaiczyk: Homology between the primary structure of α-fetoprotein, deduced from a complete cDNA sequence and serum albumin. Nature 291: 201-205, 1981.

Annual Report of the
Laboratory of Molecular Hematology
National Heart, Lung, and Blood Institute
October 1, 1981 to September 30, 1982

The Laboratory of Molecular Hematology (LMH) studies the basic molecular mechanisms of gene expression and protein synthesis, specifically using hemoglobin as a model system. LMH is closely associated with the Clinical Hematology Branch (CHB) and collaborates on a number of joint projects. LMH is composed of three segments: the Section on Molecular Genetics, which is primarily concerned with the molecular control of eukaryotic gene expression; the Section on Molecular Cloning, which is primarily concerned with the isolation and characterization of globin and other genes from the genomes of eukaryotic cells; and the Section on Protein Biosynthesis, which is primarily concerned with the mechanism and regulation of hemoglobin synthesis at the translational level.

SECTION ON MOLECULAR GENETICS AND SECTION ON MOLECULAR CLONING

The immediate objectives of these Sections are to: (1) identify, isolate and characterize the regulatory factors of animal and human DNA which are involved in the control of the expression of the globin genes; (2) analyze the genomic DNA sequences involved in the regulation of gene expression in humans and animals; and (3) develop methods for transferring functional genes into tissue culture cells and intact animals. Information from these programs will be used to study the regulation of globin gene expression in normal and thalassemic DNA. The long-term goal is to develop means whereby globin gene expression can be corrected in patients with β-thalassemia and other diseases involving abnormal hemoglobin biosynthesis.

Regulatory factors controlling the expression of the globin genes have been identified by a combination of cell biology and molecular biology techniques. Somatic cell hybrids, obtained by fusion of human or animal cells with mouse erythroleukemia (MEL) cells, have been used to provide evidence for positive and negative regulatory factors controlling globin gene expression. In order to purify and characterize these putative regulatory factors, intact-cell and cell-free assays have been established.

Besides somatic cell hybridization, several other methods of gene transfer are being used to insert genes into tissue culture cells and mouse embryos. The most successful of these has been the technique of physical microinjection in which a single copy of a specific gene can be injected into the nucleus of a single cell under conditions whereby the injected cell (or fertilized egg) can be grown in culture (or in a surrogate mother) into a cloned population (or a living animal). This technique was successfully used to correct a mouse thymidine kinase (TK) negative L cell by microinjection of a bacterial plasmid containing a functional TK gene.

Recombinant DNA technology has been used to construct "expression vectors" containing the globin gene regulatory regions. These vectors are plasmids designed to encourage expression of the inserted gene. They contain various regulatory and control regions as well as a coding sequence.

The Sections have succeeded, during the past year, in demonstrating that:

(1) Cloned genes can be microinjected into mouse fertilized eggs. In some cases the microinjected cloned gene is incorporated into the genome of the mouse, and might even be transmitted to the mouse's offspring. Attempts to cure the thalassemic defect in α-thalassemic mice are now underway using microinjected cloned α-globin genes.

(2) Expression vectors can be made containing regulatory and enhancing sequences which allow expression of globin promoter sequences in tissue culture cells and, possibly, in intact mice.

(3) The transcriptional factors required for the initiation of transcription from cloned DNA templates in a cell-free transcription system appear to be interchangeable between mouse (MEL cells), calf (calf thymus) and human (HeLa) cells.

SECTION ON PROTEIN BIOSYNTHESIS

Regulation of gene expression at the level of messenger RNA translation is an important aspect of interferon-mediated antiviral activity, cell growth, differentiation and the overall coordination of cell metabolism. Translational regulation is frequently mediated by the covalent modification of existing protein factors required for protein synthesis. It is the objective of this Section to: (1) determine and characterize the molecular basis of translational regulation; (2) identify and characterize protein and nucleic acid components participating in these regulatory mechanisms; and (3) examine the regulation of globin gene expression in normal and disease (e.g., thalassemic) states.

During the past year, the Section has successfully:

(1) Identified the molecular mechanism of translational inhibition resulting from phosphorylation of eIF-2.

(2) Purified to near homogeneity a new polypeptide complex, RF, required for GTP:GDP exchange on eIF-2.

(3) Developed sensitive radioimmunologic techniques for the study of the <u>in vitro</u> and <u>in vivo</u> distribution of eIF-2.

(4) Identified the basis of altered eIF-2 phosphatase specificity in unfractionated lysates and in highly fractionated systems.

(5) Developed microcarrier tissue culture procedures for large scale culture of CV-1 cells. These will be used for the preparation of active translational and transcriptional systems for the correlation of structure and function.

(6) Developed techniques for the quantitative analysis of various transformation procedures with the goal of increased efficiency of exogenous gene expression in cells.

(7) Determined the concentration of eIF-2 in rabbit reticulocyte lysate to be 30 to 50 pmol per ml using immunochemical techniques employing [^{125}I]protein A.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02212-06 MH

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Molecular Control of Eukaryotic Gene Expression

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	B. Safer	Medical Officer	H NHLBI
	E. Tolunay	Visiting Fellow	MH NHLBI
	Y. Chiang	Chemist	MH NHLBI
	W. Kemper	Chemist	MH NHLBI
	L. Yang	Biologist	MH NHLBI

COOPERATING UNITS (if any)
Dr. Timothy Ley, CHB, NHLBI.

LAB/BRANCH
Laboratory of Molecular Hematology

SECTION
Section on Molecular Genetics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.7	1.2	2.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to study the regulation of the globin genes at the molecular level. The approach is to identify regulatory factors which influence globin gene expression. Transcriptional regulatory factors have been isolated in crude form from mouse erythroleukemia cells and have been compared with similar fractions from HeLa cells and calf thymus. The factors apear to be interchangeable between these tissue types.

Objectives:

The objective of this project is to isolate the factors involved in the expression of eukaryotic genes (specifically, those for hemoglobin) and to understand how they interact with the regulatory sequences of DNA in order to control gene expression within the intact cell. We have shown that mouse erythroleukemia (MEL) cells, which are used as a model system for red blood cell differentiation, contain a positive regulatory factor involved in globin gene expression. The procedure is to fractionate these cells into various components which can be individually tested in cell-free transcription assays for their ability to alter mRNA synthesis.

Methods:

1) Cells are grown under standard tissue culture conditions. MEL cells are fused by means of inactivated Sendai virus (or polyethylene glycol) to other somatic cells which normally are either capable or incapable of synthesizing hemoglobin. Chromosome and isozyme analyses are done by standard cell biology techniques.

2) Cell hybrids are analyzed for their ability to synthesize globin messenger RNA (mRNA) or globin. Globin mRNA is detected and/or characterized by hybridization with the appropriate complementary DNA (cDNA) and by Berk-Sharp S1 analysis; the presence of globin is detected by radioactive leucine incorporation followed by either electrophoresis or column chromatography.

3) The cell-free transcription assay described by Roeder and his colleagues has been modified for use with MEL cell extracts. Standard protein fractionation techniques are used to isolate individually-active MEL cell fractions.

Major Findings:

1) An homologous cell-free transcription system for the mouse β-globin gene has been developed from 2S MEL cells. Extracts from uninduced MEL cells initiate transcription of other genes which encode mRNAs as well as mouse β-globin. Therefore, it appears to be a general transcription system. Three fractions, containing transcription initiation factors, have been isolated from uninduced MEL cell extracts. These fractions are being purified in order to identify and characterize individual transcription initiation factors. To isolate the factor(s) which regulates transcription of the mouse β-globin gene, extracts from induced MEL cells have been prepared and are being tested for regulatory activity.

2) Equivalent fractions from extracts of MEL cells, HeLa cells and calf thymus were compared and found to be interchangeable for accurate transcription. This suggests that the basic transcription machinery is similar in these systems and probably in all higher eukaryotes.

Major Findings: (continued)

3) Somatic cell hybrids (M11X-2, M11X-3) selectively retain human chromosome 11. These MEL hybrids were obtained by fusing HGPRT$^-$ 2S MEL cells with human fibroblasts containing and X-11 translocation, and then growing the hybrid cells in HAT selective medium. The X portion of the chimeric chromosome contains the HGPRT gene, permitting survival in HAT medium, and the 11 portion contains the β-globin gene as demonstrated by Southern blot analysis. The DNA sensitivity and methylation patterns of the human globin genes in these hybrid cells is under investigation. 2S MEL DNA methylation studies showed that no detectable change in the patterns of DNA methylation around β-globin genes was observed during HMBA-mediated erythroid differentiation.

4) M11X-2 and -3 produce human β-globin mRNA but no human β-globin polypeptide. Human β-globin mRNA was mapped by Berk-Sharp S1 mapping analysis. The message is normally 5' capped, processed and initiated. The poly A addition site also exists. It was shown that the HMBA induction of M11X-2 hybrids is transcriptionally controlled (the amount of mRNA detected increased with induction time). Using Sanger's dideoxynucleotide sequencing method, part of the normal human mRNA was sequenced using specific synthetic DNA oligonucleotides. The human mRNA in the hybrid cells will now be sequenced.

Significance to Biomedical Research and Institute Program:

The molecular control of eukaryotic gene expression remains one of the major questions in biology today. Once it is better understood how a gene in a eukaryotic cell is controlled, this knowledge can be applied to a wide range of human diseases including genetic diseases, viral diseases, etc.

Proposed Course of Project:

Purification and characterization of the transcription initiation factors and fractionation of extracts from induced MEL cells in order to isolate the factor(s) which regulate the expression of mouse β-globin gene are proposed. The biologcial function of the individual factors will be studied, both in regard to their ability to interact with DNA and their ability to control the regulation of gene expression. The mechanism of the initiation of transcription will be studied in crude and partially purified extracts in order to understand the order of events during initiation of transcription. The structure and function of the abnormal human β-globin mRNA in the somatic cell hybrids M11X-2 and -3 will continue to be studied.

Publications:

1. Vembu, D., Young, N.S., Willing, M., Church, E., Sanders-Haigh, L., and Anderson, W.F.: Regulation of human globin gene expression in mouse erythroleukemia x human fibroblast cells. Somat. Cell Genet. 8: 163-178, 1982.

2. Tolunay, H. E., Yang. L., Kemper, W.M., Safer, B. and Anderson, W.F.: An homologous globin cell-free transcription system with comparison of heterologous factors. J. Biol. Chem. 1982, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02213-05 MH

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Regulation of Protein Biosynthesis in Cell-Free Systems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Brian Safer	Medical Officer	MH NHLBI
OTHER:	Rosemary Jagus	Visiting Associate	MH NHLBI
	Therese Brendler	Staff Fellow	MH NHLBI
	Deborah Crouch	Biologist	MH NHLBI
	Eve Church	Microbiologist	MH NHLBI
	Millie Schafer	Chemist	MH NHLBI

COOPERATING UNITS (if any) P. Cohen, University of Dundee, Dundee, Scotland; I.G. Wool, University of Chicago, Chicago, Illinois; P. Torrence, Laboratory of Chemistry, NIAMDD; S.G. Chaney, University of North Carolina, Chapel Hill, North Carolina.

LAB/BRANCH
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SECTION
Section on Protein Biosynthesis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.8	1.0	1.8

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Regulation of gene expression at the level of mRNA translation is important to rapid alteration of cell metabolism in response to stress. In eukaryotes, regulation of protein synthesis is frequently achieved by the rapid and reversible covalent modification of proteins required for the initiation of translation. This laboratory has studied the role of eIF-2 phosphorylation and its relationship to the accompanying inhibition of protein synthesis. The phosphorylation state of eIF-2 is closely regulated not only by the activity of its specific kinase and protein phosphatase, but also by binding of its ligands, GTP and Met-tRNAi and by association with a new polypeptide complex which we designate RF. The major function of RF is to catalyze the exchange of GTP for GDP in the inactive eIF-2.GDP complex, required for reactivation of eIF-2 following each round of initiation. In hemin-deficient lysate, a limited phosphorylation of eIF-2 (20-30%) is nevertheless able to almost totally inhibit protein synthesis by sequestering the smaller RF pool into an inactive RF.eIF-2(αP) complex, incapable of guanine nucleotide exchange. In addition to alterations of kinase activity, regulation of the phosphorylation state of eIF-2 is controlled by ligand-dependent restrictions on the access of phosphatase to phosphorylation sites on eIF-2. f02

Objectives:

The major goals are: (1) to determine the sites and mechanisms of translational control of protein synthesis in vitro; (2) to identify and characterize the components involved in such regulation; (3) to compare and examine translational control mechanisms in reticulocyte lysate and CV-1 cells; (4) to develop active in vitro translation and transcription systems which retain key regulatory systems found in intact cells; (5) to examine the relationships of translational and transcriptional components to cell ultrastructure; and (6) to develop more efficient transformation systems for expression of cloned genes.

Methods:

Conventional chromatographic procedures and affinity chromatography using antibodies prepared against purified initiation factors will be used to isolately rapidly key translational and transcriptional components under defined experimental conditions. Covalent modification of these regulatory factors will be examined by incorporation of specific radioisotopes, direct chemical analogs, group specific reagents, and by alteration of their physical characteristics (e.g., pI, $S_{20,w}$). Ligand binding to preinitiation complexes formed during protein synthesis initiation are examined by Scatchard analysis of equilibrium binding data. Active translation and transcription systems will be developed for CV-1 cells using non-disruptive procedures which maintain cell ultrastructure. Large scale culture of CV-1 cells on microcarrier beads will be used to obtain the large amounts of cells required for purification of translational and transcriptional components. Cell ultrastructure will be examined by transmission electron microscopy.

Major Findings:

1. Inhibition of protein synthesis initiation in hemin-deficient reticulocyte lysate results from the inhibition of the guanine nucleotide exchange mechanism for eIF-2.

2. Exchange of bound GDP (from inactive eIF-2•GDP complex) for GTP, required for binding of the initiator tRNA species methionyl-$tRNA_i$, is mediated by formation of a 390,000 dalton polypeptide complex of a new initiation factor RF and eIF-2. Guanine nucleotide exchange at physiologic ratios of GTP and GDP is the result of an increased K_D^{GDP} (from $3.x\ 10^{-8}$M to 2.2×10^{-7} M) of eIF-2 upon formation of the RF•eIF-2 complex.

3. The RF•eIF-2 complex has been purified 2000-fold to near homogeneity. The RF•eIF-2 complex has 8 distinct subunits. High salt or phosphorylation of the eIF-2α subunit dissociate the complex into free RF and eIF-2. The 80 M_r subunit is a phosphoprotein. The possible functional significance of this phosphorylation site is being explored.

Major Findings (continued)

4. Phosphorylation of eIF-2 by the hemin-regulated eIF-2α kinase does not directy inactivate eIF-2 Met-tRNA$_i$ binding activity. Rather, at physiologic salt and guanine nucleotide concentrations, RF and eIF-2(αP) form a tight complex which is unable to effect exchange of GTP for bound GDP. Thus, catalytic recycling of eIF-2 during protein synthesis initiation is inhibited. This exchange mechanism can be bypassed by adding sufficient GTP to allow exchange with bound GDP by mass action.

5. The relative pool sizes of eIF-2 and RF has been determined to be 5:1 by isotopic and immunologic methods. The sequestration of RF into an inactive RF•eIF-2(αP) pool following eIF-2 phosphorylation can now account for the nearly total inhibition of activity in hemin-deficient lysate, even though only 25 percent of the eIF-2 pool is phosphorylated.

6. The mechanisms of inhibition produced by double stranded RNA in interferon-treated mouse L-cell and reticulocyte lysates were compared. In both systems, dsRNA activates a specific protein kinase for eIF-2 and 2'-5' oligoA synthetase, which leads to activation of RNase F. In contrast to reticulocyte lysate where phosphorylation of eIF-2α appears to be the primary inhibitory mechanism, translation in L-cell extracts is not inhibited by eIF-α phosphorylation, but involves mRNA degradation by RNase F activation.

7. Regulation of eIF-2 phosphatase activity against sites on the α and β subunits is regulated by changes in the functional state of eIF-2. Formation of the RF•eIF-2 complex restricts access of the phosphatase to both the α and β subunit phosphorylation sites. In the eIF-2•Met-tRNA$_i$•GTP ternary complex, eIF-2α can be rapidly dephosphorylated, but accessibility to the β subunit phosphorylation sites is totally blocked. In contrast, both the α and β subunits of eIF-2 are rapidly dephosphorylated in free eIF-2. Since association of free eIF-2 with RF or Met-tRNA$_i$ is regulated by its sulfhydryl group redox state or guanine nucleotide energy state, respectively, control of phosphatase activity can be finely regulated by a variety of factors which control its access to phosphorylation sites of eIF-2.

Significance to Biomedical Research and Institute Program:

Although regulation at the level of transcription is currently thought to be the primary mechanism for regulating the flow of genetic information, modulation of protein synthesis and interactions between translational and transcriptional components have recently assumed increased importance. Translational regulation has been identified as a major feature of host virus interaction. Regulation of protein synthesis by hemin was once thought to be a highly specialized means of coordinating globin chain and hemin synthesis in reticulocytes; it now appears, however to be a widespread mechanism for post-translational modulation of gene expression involving a cascade of highly specific protein kinases and other covalent modifiers. Final control of gene expression during cell differentiation may also be regulated by interaction of translational components with messenger RNA, which, in turn, may interact with and be regulated by changes in the ultrastructure of the cell. It is essential, therefore, to understand the basic mechanisms involved in these processes to be able to control gene expression in the cell.

Proposed Course of the Project:

Peptides containing the phosphorylation sites of the eIF-2α and β subunits are being purified and sequenced. The amino acid sequence will be used to construct DNA probes for the isolation of eIF-2 mRNAs and complete sequencing of these subunits. This will allow more detailed studies of the molecular basis of eIF-2 regulation.

Antibodies produced against eIF-2α and β will be purified by affinity chormatography and used to probe alterations of eIF-2 function in unfractionated translation systems. The function of other factors, both translational and transcriptional will be explored by similar procedures.

New translation and transcription systems are being developed using a variety of techniques which minimally disrupt cell ultrastructure. Correlations between alterations of structure and function will be drawn by comparing functional changes with structural effects observed with high voltage transmission electron microscopy.

Regulation of gene expression, both transcriptional and translational, will be examined in CV-2 cells. Studies are currently underway to optimize expression of foreign genes in this system.

Publications:

1. Jacobsen, H., Epstein, D.A., Friedman, R.M., Safer, B., and Torrence, P.F.: dsRNA-dependent phosphorylation of protein P1 and eIF-2 does not correlate with protein synthesis inhibition in cell free systems from IFN-treated cells. Proc. Natl. Acad. Sci. USA, 1982.

2. Torrence, P.F., Imai, J., Lesiak, K., Johnson, M.I., Jacobsen, H., Friedman, R.M., Sawai, H., and Safer, B.: Double stranded RNA and 2',-5'-oligoadenylates: Comparisons in interferon actionπ In Merigan, T., Friedman, R., and Fox, C. Fred (Eds.): Chemistry and Biology of Interferons: Relationship to Therapeutics, UCLA Symposia on Molecular and Cellular Biology. Vol. 25, New York, Academic Press, 1982, in press.

3. Safer, B., Jagus, R., Konieczny, A., and Crouch, D.: The mechanism of translational inhibition in hemin-deficient lysates. In Grunberg-Manago, M., and Safer, B. (Eds.): Translational and Transcriptionl Interactions during the Regulation of Gene Expression. New York, Elsevier, 1982.

4. Safer, B., and Jagus, R.: New developments in the regulation of eIF-2. Biochimie 643: 709-717, 1981.

5. Safer, B., Jagus, R., and Crouch, D.: Oxidation/reduction and phosphorylation states of eIF-2 both regulate protein synthesis initiation. In Cold Spring Harbor Conferences on Cell Proliferation, Vol.8: Protein Phosphorylation. 1981, pp. 979-998.

Publications (continued)

6. Chan, Y.-L., Ulbrich, N., Ackerman, E.J., Todokoro, K., Slobin, L.I., Safer, B., Sigler, P.B., and Wool, I.G.: The binding of transfer ribonucleic acids to 5S and 5.8S eukaryotic ribosomal ribonucleic acid-protein complexes. J. Biol. Chem. 257: 2522-2527, 1981.

7. Pato, M.D., Adelstein, R.S., Crouch, D., , Safer, B., Ingebritsen, T.S., and Cohen, P.: Classification of two homogeneous myosin light chain phosphatases from smooth muscle as protein phosphatase $2A_1$ and 2C, and a homogeneous protein phosphatase from reticulocytes active on initiation factor eIF-2 as protein phosphatase $2A_2$. Eur. J. Biochem., in press, 1982.

8. Safer, B., Jagus, R., Konieczny, A., and Crouch, D.: Catalytic utilization of eIF-2: Regulation by covalent modification and recycling factors in regulation of hemoglobin synthesis. In Goldwasser, E., (Ed.): Regulation of Hemoglobin Biosynthesis, New York, Elsevier, in press, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02214-05 MH

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Molecular Cloning of Eukaryotic Globin Gene Sequences

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. French Anderson	Chief	MH NHLBI
OTHER:	P. Berg	Expert	MH NHLBI
	Z. Popovic	Guest Worker	MH NHLBI
	J.-K. Yu	Visiting Fellow	MH NHLBI
	L. Hildebrand	Medical Technologist	MH NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Molecular Hematology

SECTION
Section on Molecular Cloning

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.7	1.2	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to apply recombinant DNA technology to the analysis of the mouse β-globin-maj 5' flanking DNA sequences. Various plasmids have been constructed which will allow us to study the effect of DNA enhancing sequences on activity of the β-globin promoter, both in tissue culture cells and in whole mice. These are called expression vectors. We hope to be able to identify the DNA region required for induction of β-globin synthesis using these plasmids in cultured erythroid cells and in intact mice.

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(Rev. 2-81)

Objectives:

This project has three objectives: (1) to study the effect of enhancer sequences on expression from the mouse β-globin-maj gene promoter in order to maximize gene expression and learn more about the specificity of enhancers; (2) to identify the DNA sequence(s) necessary for induction of β-globin synthesis in mouse erythroleukemia (MEL) cells; and (3) to study activity of the cloned α-globin and β-globin promoters in cultured cells and in mice.

Methods:

1. The 5' flanking region including the promoter of the mouse β-globin-maj gene has been cloned into an eucaryotic expression vector developed by Martin Rosenberg (NCI). This promoter now directs expression of the galactokinase gene from E. coli.

2. Two enhancer sequences have been cloned into this plasmid singly and in combination. They are the 72 base pair repeats from SV40 and the 74 base pair repeats from Harvey sarcoma virus.

3. Activity of galactokinase is assayed using a transient expression assay. This involves introducing plasmid DNA into monkey kidney cells (CV-1 cells) or mouse cells (L cells) in culture, then assaying galactokinase by starch gel electrophoresis.

4. Induction of the β-globin promoter is assayed by introducing the expression vector plasmid DNA into MEL cells and measuring galactokinase in induced and uninduced cells.

5. Plasmid DNA has been microinjected into fertilized mouse oocytes which were then implanted in pseudopregnant females. The presence of the plasmid was determined by Southern blotting of DNA from the tails of the offspring.

Major Findings:

1. The expression of galactokinase using the mouse β-globin promoter is dependent on the presence of an enhancer in CV-1 cells. Assays in L cells are currently underway.

2. The relative strength of the β-globin promoter has been compared with that of the SV40 early promoter by assaying galactokinase synthesis directed by two comparable plasmids. The β-globin promoter exhibited 16% as much activity as the SV40 promoter in CV-1 cells.

Major Findings (Continued)

3. A comparison is underway of the following plasmids in both CV-1 cells and L cells:

Promoter	Enhancer
β-globin	SV40
	Harvey
	Both
SV40	SV40
	Harvey
	Both

We expect the enhancer from SV40 to work better in CV-1 cells and the enhancer from Harvey sarcoma virus to work better in L cells, since CV-1 cells are permissive for SV40 and L cells are permissive for Harvey sarcoma virus.

4. MEL cells are being transformed with plasmid DNA using the calcium phosphate method and will be induced for β-globin synthesis using a chemical inducer. This will allow us to determine whether the cloned β-globin promoter is activated by inducers, since we can assay galactokinase in induced and uninduced MEL cells.

5. Of 16 mice born after microinjection, 2 were positive for the presence of the expression vector plasmid DNA. One died at birth and the other (a male) has been mated with wild type females. Several females are pregnant and several others have delivered. Spleen cells from the original mouse will be assayed for galactokinase activity as soon as offspring positive for the presence of the plasmid have been identified.

Significance to Biomedical Research and Institute Program:

This work should increase our understanding of gene expression both at the molecular level and the organismic level. The DNA sequence(s) important for induction of the β-globin gene will be identified and the effect of enhancer sequences on gene expression will be studied. The introduction of a cloned gene into a mouse has been accomplished and studies on the expression of this gene, using a mouse promoter, will yield valuable information leading towards the eventual gene therapy of humans.

Proposed Course of the Project:

Various constructs described above will be used to look at induction in MEL cells. The plasmid giving the maximum difference in expression between induced and uninduced cells can then be used to localize the DNA necessary for induction. After this DNA region is identified, it will be studied using mutagenesis techniques. Furthermore, some of these plasmids will be introduced into mice to study possible relationships between plasmid structure and gene function in the whole animal.

Publications:

1. Kretschmer, P.J., Bowman, A.H., Huberman, M.H., Sanders-Haigh, L., Killos, L., and Anderson, W.F.: Recovery of recombinant bacterial plasmids from E. coli transformed with DNA from microinjected mouse cells. Nucl. Acids Res. 9: 6199-6217, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02216-03 MH

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Correction of Genetic Defects by Gene Transfer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. French Anderson	Chief	MH NHLBI
OTHER:	P. Berg	Expert	MH NHLBI
	S. Bernstein	Biologist	MH NHLBI
	J. DiPietro	Biologist	MH NHLBI
	K. Humphries	Visiting Associate	CH NHLBI
	A. Nienhuis	Chief	CH NHLBI

COOPERATING UNITS (if any)

E.G. Diacumakos, Ph.D., Rockefeller University, New York, New York

LAB/BRANCH

Laboratory of Molecular Hematology

SECTION

Section on Molecular Genetics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.2	0.9	1.3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Methods have been developed for transferring functional genes into mammalian cells. A primary technique is physical microinjection of specific cloned genes into the nucleus of individual tissue culture cells and into mouse fertilized eggs. Adult mice containing microinjected cloned genes in their genomic DNA have been obtained.

£11

Objectives:

The objective of this project is to develop methods for transferring functional genes into mammalian tissue culture cells and into intact animals. Ultimately, the techniques would be used for attempting to correct genetic diseases in human patients.

Methods:

1) Tissue culture cells are grown under standard tissue culture conditions.

2) Plasmids containing specific cloned genes are made by standard recombinant DNA techniques.

3) Physical microinjection into specific regions of intact mammalian cells is carried out by using ultra-thin micropipettes under phase optics according to the procedure of Diacumakos (Diacumakos, E.G., Methods in Cell Biology 7: 287-311, 1973). Single cell clones are grown to 10^8-10^9 cells for isolation of DNA and RNA for analysis by restriction endonuclease and Southern blot analysis, by mRNA-cDNA liquid hybridization, or by Northern blot analysis.

4) Gene transfer to mice by microinjection of fertilized eggs involves several specialized techniques which are briefly described below.

(a) Collection of zygotes: C57BL/6J females are mated to LT/SV males. Females with vaginal plugs (day 1 of pregnancy) are sacrificed and fertilized eggs at the pronuclear stage are harvested from oviducts. Eggs are freed from follicle cells by mild enzyme treatment and held in special medium in a controlled atmosphere until microinjection.

(b) Microinjection and micromanipulation of eggs: Precise injection pipettes with an external diameter of 1 μ are drawn on a vertical pipette puller. Holding pipettes of 60 to 70 μ in diameter are drawn by hand. With a Zeiss inverted microscope and Leitz micromanipulators, eggs, in a small drop of media under oil, are positioned on the holding pipette so that the male pronucleus is in juxtaposition to the injection pipette. The injecting pipette is then inserted into the male pronucleus and approximately 10 pl of DNA solution is introduced.

(c) Embryo culture and transplantation: After microinjection, zygotes are cultured to observe their preimplantation development for a period of 4 days. Eggs normally developing to morula stage are then transplanted into the uterae of day-3 pseudopregnant B6D2F1/J hybrid foster mothers. After 20 days gestation, mice are delivered spontaneously or, if necessary, by Caesarean section.

(d) Screening for donor DNA in mice: In most cases for screening, high molecular weight DNA is prepared from a small portion of tail obtained at 2 weeks of age. DNA is analyzed in Southern blots or spot blots using radioactive probes prepared from the injected DNA sequences.

(e) Analysis of germ line transmission and gene expression: Positive mice are mated and offspring screened for evidence of germ line transmission of donor DNA sequences. Gene expression is tested in a variety of tissues using sensitive assays for protein and/or RNA. Further studies to characterize the nature of the donor DNA will include: restriction enzyme analysis to determne the methylation status and *in situ* chromosome hybridization to determine the site of integration.

Major Findings:

1) Much of the past year has been spent in acquiring the necessary technical expertise to generate reliably the necessary numbers of eggs, to fabricate precision and holding pipettes, and to develop conditions for embryo culture and transfer. A total of five different phage or plasmid recombinants containing human or mouse globin gene segments have now been used as a source of donor DNA for microinjection.

2) A total of 50 mice have been born from microinjected eggs; 46 mice have reached adulthood, 4 mice were stillborn or died shortly after birth. Of these 50 mice, 3 have been found to contain donor DNA sequences (2 live mice, 1 stillborn). Positive mice are now being mated to determine if there is germ line transmission of donor DNA; when suitable numbers of offspring are available, parent mice and offspring will be analyzed for gene expression.

Significance to Biomedical Research and Institute Program:

The long-term aim of much of the work in molecular genetics is to develop techniques for treating or curing human genetic defects. This project utilizes recombinant DNA technology, mouse genetics and embryology, and cell biology techniques to try to accomplish this goal.

Proposed Course of the Project:

Analysis of genes microinjected into mouse eggs and into erythroid cells (viz. mouse erythroleukemia cells) will continue in order to understand regulation of the globin gene locus. Microinjection of eggs from thalassemic mice with globin genes will be carried out in an attempt to cure a genetic defect (thalassemia) *in vivo*.

Publications:

1. Anderson, W.F.. Killos, L., Sanders-Haigh, L., Kretschmer, P., Diacumakos, E., Nienhuis, A., Willing, M., and Vembu, D.: Regulation of human globin gene expression after gene transfer. In Sigler, P.B. (Ed.): The Molecular Basis of Mutant Hemoglobin Dysfunction (University of Chicago Sickle Cell Center Hemoglobin Symposia, Vol. 1). New York, Elsevier North-Holland, 1981, pp. 79-96.

Publications (continued)

2. Anderson, W.F.: Expression of microinjected eucaryotic genes. In Niu, M.C., and Chuang, H.H. (Eds.): The Role of RNA in Development and Reproduction (Second International Symposium). New York, Van Nostrand Reinhold Co., 1981, pp. 29-48.

3. Martinell, J., Whitney, J.B. III, Popp, R.A., Russell, L.B., and Anderson, W.F.: Three mouse model of human thalassemia. Proc. Natl. Acad. Sci. USA 78: 5056-5060, 1981.

4. Popp, R.A., Lalley, P.A., Whitney, J.B., III, and Anderson, W.F.: Mouse α-globin genes and α-like pseudogenes are not syntenic. Proc. Natl. Acad. Sci. USA 78: 6362-6366, 1981.

5. Whitney, J.B., III, Martinell, J., Popp, R.A., Russell, L.A., and Anderson, W.F.: Deletions in the α-globin gene complex in α-thalassemic mice. Proc. Natl. Acad. Sci. USA 78: 7644-7647, 1981.

6. Anderson, W.F.: Gene therapy (Bench and Bedside Series). J.A.M.A. 246: 2737-2739, 1981.

7. Anderson, W.F., Martinell, J., Whitney, J.B., III, and Popp, R.A.: Mouse models of human thalassemia. In Desnick, R.J. (Ed.): Animal Models of Inborn Errors of Metabolism. New York, Alan R. Liss, Inc. 1982, pp. 11-26.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02218-02 MH

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Regulation of eIF-2 Activity.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Rosemary Jagus	Visiting Associate	MH NHLBI
OTHER:	Brian Safer	Medical Officer	MH NHLBI
	Deborah Crouch	Biologist	MH NHLBI
	Eve Church	Microbiologist	MH NHLBI

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NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	1.0	0.6

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Eukaryotic initiation factor 2, eIF-2, plays a regulatory role in the initiation of protein synthesis in animal cells. eIF-2 activity is modulated by phosphorylation, guanine nucleotides and the redox state of its sulfhydryl groups and has been used as a model protein in which to study these important regulatory mechanisms of animal cell function. Phosphorylation of the α-subunit, which leads to the inhibition of protein synthesis, is caused by a defect in the interaction of eIF-2 with RF, a protein involved in guanine nucleotide exchange on eIF-2. Elucidation of the mechanism of inhibition has been hampered by uncertainties concerning eIF-2 subunit composition and function, the size of the eIF-2 pool, an imperfect understanding of eIF-2 distribution under different protein synthetic conditions and a lack of appreciation of the proteins with which eIF-2 normally associates in vivo. Immunochemical techniques sensitive to 0.003 μg have been used to quantitate eIF-2 and RF levels in reticulocyte lysate and to monitor eIF-2 distribution among the intermediate preinitiation complexes under a variety of physiologic states.

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PHS-6040
(Rev. 2-81)

Objectives:

The major objectives are: (1) to quantitate the levels of eIF-2 and RF in reticuloyte lysate; (2) monitor eIF-2 localization with respect to RF and other components of initiation under different protein synthesis conditions and determine the mechanism by which phosphorylation of the α-subunit of eIF-2 inhibits protein synthesis; and (3) extend these studies to nucleated cells to determine whether the type of regulation of eIF-2 activity observed in reticulocyte lysate is employed in other cell types and to assess its significance in the quantitative control of protein synthesis.

Methods:

eIF-2 and RF levels are quantitated by an immunochemical technique involving separation of lysate proteins by one-dimensional SDS-PAGE, followed by electrophoretic transfer of proteins to nitrocellulose (Western blot) and visualization of eIF-2 and RF subunits using antibodies (conventional and monoclonal) and [^{125}I]protein A. The same technique is used to determine eIF-2 and RF localization after sucrose density gradient fractionation of incubated lysate. The phosphorylation state of eIF-2 is monitored by combining the immunochemical determination of eIF-2 concentration with one-dimensional SDS-PAGE resolution of the α-subunit of eIF-2 by a modified Laemmli technique utilizing higher bis:acrylamide ratios and a pH of 9.1. This method has been adapted to monitor <u>in situ</u> dephosphorylation rates of endogenous eIF-2.

Major Findings:

1. The concentration of eIF-2 in lysate is found to be between 0.175 - 0.25 pmol/pmol 80S ribosome equivalents. Approximately 25-30% of eIF-2 is found in lysate in a complex sedimenting at 20-25S. The eIF-2 in this complex may be in the RF.eIF-2 form.

2. One of the RF subunits, an 80,0000 dalton polypeptide, is found in stoichiometric amounts with eIF-2, although less direct evidence suggests an eIF-2:RF ratio at 5:1. The behavior of the 80,000 dalton subunit during sucrose density gradient fractionation of lysate suggests that the pool of 80,000 dalton subunit not associated with RF.eIF-2 is present as a ribosomal bound element.

3. Dramatic changes in eIF-2 distribution have not been observed under different protein synthesis conditions. Relatively low levels (approximately 10%) of eIF-2 are found free; approximately 60% is ribosome bound and approximately 30% is found in a 20-25S complex.

4. Rates of dephosphorylation of the endogenous α-subunit of eIF-2 in lysate are found to be very rapid (t 1/2 = 1.5'), but slower than the dephosphorylation rate of [^{32}P]eIF-2 added to lysate (t 1/2 = 20-30 secs). This difference in dephosphorylation rate is thought to reflect the fact that approximately 90% of endogenous eIF-2 is found associated in large protein complexes or bound to ribosomes.

Significance to Biomedical Research and Institute Program:

Translational regulation represents the short-term regulation of gene expression found in animal cells and the mechanisms available are exploited during development, differentiation, transformation and by many animal cell viruses, making it important to understand the basic mechanisms involved. GTP-modulated regulatory proteins are an increasingly common element in animal cell regulation. Such proteins play roles in the hormonal control of adenylate cyclase, in information amplification in the visual response to rhodopsin and the maintenance of the transformed state by retroviruses. eIF-2, in its regulatory role in initiation, provides a useful model system in which to study modulation by guanine nucleotides.

Proposed Course of Project:

1. Studies involving the localization of eIF-2 in different initiation complexes and the effects of phosphorylation are being further pursued.

2. eIF-2 levels/ribosome and eIF-2 localization are being monitored in a variety of cell types (myelomas, human T-lymphocytes, oocytes, developing embryos) under a variety of protein synthesis conditions. The phosphorylation state of the α-subunit of eIF-2 will also be monitored.

Publications:

1. Cooper, D.M.F., Jagus, R., Somers, R.L. and Rodbell, M.: Cholera toxin modifies diverse GTP-modulated regulatory proteins. B.B.R.C. 101:1179-1185, 1981.

2. Jagus, R., Crouch, D., Konieczny, A., and Safer B: The role of phosphorylation in the regulation of eukaryotic initiation factor 2 activity. Current Topics in Cellular Regulation 21: 53-83, 1982.

3. Cooper, D.M.F., and Jagus, R.: Impaired adenylate cyclase activity of phenylhydrazine-induced reticulocytes. J. Biol. Chem. 257: 4686-4688, 1982.

Annual Report of the
Section on Laboratory Animal Medicine and Surgery
Office of the Director of Intramural Research
National Heart, Lung, and Blood Institute
October 1, 1981 to September 30, 1982

The Section functions primarily in a support role to all laboratories of IR providing care for many species of animals, technical assistance in preparation and maintenance of animal models for various experimental regimens, and the development of animal resources not otherwise available.

Maintenance of various small animal species has been accomplished in designated areas in close proximity to IR laboratories in Buildings 3, 10, and 36. Large animal species are maintained in Buildings 3, 28, the NIHAC, and at Luray, Virginia. Postoperative intensive care and treatment of surgery patients is completed in Buildings 3, 14-E, and 28.

The animal surgery laboratory located in Building 14-E supports the Laboratory of Molecular Hematology, Clinical Hematology Branch, Hypertension-Endocrine Branch, Laboratory of Technical Development, the Surgery Branch, and the Pulmonary Branch in preparation of experimental animal models, completing cardiovascular studies and in collecting various biological speciments. The laboratory operates an x-ray catheterization suite, clinical chemistry laboratory, sterile operating suites, and special study suites required to meet IR requirements.

The NHLBI Sheep Colony continues year-round breeding of laboratory sheep. Approximately 500 animals were delivered to laboratories meeting requirements of gestation stages from 22 - 140 days and various age and size lambs, young adults, and aged sheep. In addition, postoperative animal models have been returned and maintained at the colony and more than 125 tons of feed supplies have been delivered to NIH.

Laboratory studies have been underway to define hemodynamic parameters and tissue morphology resultant of Newfoundland dog left ventricular hypertrophy due to infracoronary left ventricle outflow tract obstruction. Disease processes will be characterized to determine usefulness of this unique animal model for future laboratory study.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03401 -06 LAMS

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Newfoundland Breeding Colony

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.E. Pierce	Chief	SLAMS, NHLBI
	M. Jones	Senior Surgeon	SB, NHLBI
Other:	T. Picone	Staff Fellow	SB, NHLBI
	P. Hunter	Staff Fellow	SLAMS, NHLBI

COOPERATING UNITS (if any)

Clinic of Surgery

LAB/BRANCH
Office of the Director of Intramural Research

SECTION
Section on Laboratory Animal Medicine and Surgery

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	3.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The Newfoundland Breeding Colony has been developed as a source of laboratory dogs affected with left ventricular hypertrophy (LVH) due to infracoronary left ventricle outflow tract obstruction and other spontaneously occurring heart defects. More than 150 dogs have been reared, all having some form of hereditary subaortic stenosis (SAS) and/or pulmonary outflow tract obstruction.

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PHS-6040
(Rev. 2-81)

Project Description:

The Newfoundland Breeding Colony was maintained by Flow Laboratories, Inc., at Dublin, Virginia supported by NIH 263-78-D-0253. Fifty-six dogs are maintained in Building 28.

Availability of a naturally occurring animal model for study of LVH resultant of infracoronary LV outflow tract obstruction is important because technical difficulties have not been satisfactorily overcome in attempts to produce such a defect in normal animals.

Discrete subaortic stenosis has been studied in Newfoundland dogs at the School of Veterinary Medicine, University of Pennsylvania where initial breeding experiments suggested that it is inherited and either a polygenic or an autosomal dominant trait with modifiers.

Breeding is continuing to produce offsprings to specifically monitor hemodynamic patterns during development of varying abnormalities over several years.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03402 -06 LAMS

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

NHLBI Laboratory Sheep Colony

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J.E. Pierce Chief SLAMS, NHLBI

COOPERATING UNITS (if any)

1. Laboratory of Biomedical Sciences, IRP, NICHHD
2. ACS, VRB, DRS

LAB/BRANCH

Office of the Director of Intramural Research

SECTION

Section on Laboratory Animal Medicine and Surgery

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.8	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The Laboratory Sheep Colony is an NIH animal resource providing varied age sheep that meet specific year-round requirements of the Clinical Hematology Branch, Laboratory of Technical Development, Pulmonary Branch, and Surgery Branch, DIR, NHLBI; and the Laboratory of Biomedical Sciences, IRP, NICHHD. Maintenance regimens in use have resulted in successful year-round breeding and production of varied age sheep.

Practices that have contributed to reduction of undesired seasonal variables include: (1) continuous prophylactic immunization of all age animal groups; (2) accurate pregnancy diagnosis during first trimester using Doppler ultrasound: (3) monitoring of animal health using various diagnostic laboratory techniques; and (4) many husbandry techniques unique to this colony. Such practices have been cost prohibitive in commerical sheep flocks that result in inconsistent availability and existence of varied states of health in animals delivered for laboratory use.

821

PHS-6040
(Rev. 2-81)

Project Description

The breeding colony continues as a source of sheep with A, AB, and B type hemoglobin for the CHB and meets other supportive laboratory program requiements of young lambs and pregnant ewes as required. From 800 to 950 varied age sheep have existed in the colony year-round. Approximately 500 animals were delivered to NIH and other facilities for laboratory use during the report period.

The contractor has been responsible for developing and updating husbandry techniques as instructed by the project officer that allow optimal conditions for natural year-round breeding at the contract site. The project officer has been responsible for monitoring techniques and updating guidelines followed by the contractor to meet NIH laboratory requirements along with animal health regimens (sheep property of NIH) that allow minimal susceptailibity of all age groups to common sheep diseases.

Immunization protocols direct personnel to administer specific toxoids and bacterins to lambs with biweekly boosters of each by the fifth week of age. This practice has essentially eliminated enterotoxemia and more than 90% of chronic pneumonia previosuly experienced. Repeated administration of indicated biologicals is carried out in all age groups at designated periods of development and production.

The necessity of an accurate method of pregnancy diagnosis during early gestation was determined during initial development of the colony. Natural seasonal and environmental conditions effect the conception rate varying from 0-100%. Lindahl's technique using Doppler ultrasound with rectal examination has been adequate. It allows accurate diagnosis of up to 100% of pregnant sheep from 21-35 days gestation. Examinations are performed weekly by contract personnel with more than 1,500 examinations completed per year to detect approximately 500 pregnant ewes conceived over a 52-week period.

Laboratory tests are continuously performed to monitor flock health. Contract personnel monitor internal parasite infestation by random fecal sampling from various animal groups with examination using direct and flotation methods. CBC and blood chemistry profiles are performed on animals delivered to the laboratory to monitor health and nutrition status. Microbiological and serological screening for detection of suspected disease entities is carried out when indicated.

In addition to producing sheep for laboratory use, the contractor has maintained animals for periods over 6 months and returned for study.

Also, more than 125 tons of feed supplies were delivered to NIH to allow continuation of colony animal diets in laboratory facilities.

This project will continue as IR and other NIH programs have sufficient requirements that justify the continued support of this laborartory sheep resource. Production goals and total numbers of animals maintained will be varied as required by changing demands of laboratories.

Contract Information

Contract Number:	263-80-C-0007 - approximately $280,000 - 10/1/81 - 9/30/82
Contract Site:	White House Farms, Inc. Rt. 1, Box 403-E Luray, Virginia 22835
PI:	Max Foltz, Contractor Rick Miller, Colony Manager
Total Manyears:	8.0
Professional:	2.0
Other:	6.0

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03403 -05 LAMS

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Indirect Blood Pressure Measurements in Laboratory Animals Using Oscillometry.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.E. Pierce	Chief	SLAMS, NHLBI
	E. Walker	Medical Engineer	BEI, DRS

COOPERATING UNITS (if any)

E. Walker, BEI, DRS

LAB/BRANCH

Office of the Director of Intramural Research

SECTION

Section on Laboratory Animal Medicine and Surgery

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.8	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A technique for obtaining indirect blood pressure measurements in laboratory animals is currently being evaluated. Oscillometry monitors the oscillations in cuff pressure with a pressure transducer as the cuff is deflated. During deflation from super systolic levels, the first increase in oscillation observed is designated as systolic blood pressure.

Mean arterial measurements obtained on anesthetized dogs with direct pressure measurements available were consistently within 1-3% of direct pressure.

Measurements obtained on awake dogs were not as consistent but corresponding values were obtained in both forelegs over a 15-minute examination period within 5% of each other. (Direct measurements not simultaneously available).

The technique has been further evaluated using a commerically available unit and a laboratory designated ocillometer which provides raw oscillation data to assist in determining optimal cuff sizes to be used and identify other variables that effect obtaining accurate blood pressure measurements in laboratory animals.

824

PHS-6040
(Rev. 2-81)

Project Description:

A technique for obtaining accurate indirect blood pressure measurements in laboratory animals, particularly dogs and miniature swine has been continuously requested by investigators of the Laboratory of Experimental Atherosclerosis, Hypertension-Endocrine Branch, and Surgery Branch, IR.

A technique using Doppler ultrasound with transducer secured to an inflatable cuff has proved unsatisfactory. Inconsistent blood pressure values were obtained in awake and anesthetized animals locating the cuff at various sites on forelimb, rear leg, and tail as reported successful by other groups.

Oscillometry is a method of measuring blood pressure by analyzing the pulse pattern of the cuff pressure oscillations. In practice, a cuff is placed around a limb and inflated to a pressure above systolic and then slowly deflated. While the cuff is being deflated the amplitude of oscillation in cuff pressure, produced by the arterial pulse beneath the cuff, is monitored. Systolic pressure is indicated by the first significant increase in oscillation amplitude. Diastolic pressure is indicated as the lowest cuff pressure at the point of maximum oscillation in cuff pressure. Currently, most investigators using this technique take the point of maxium oscillation to indicate mean arterial pressure.

The commerically available unit (Applied Medical Research Corporation) was found to give accurate measurements on animals in the asleep state and varied measurements in the awake state because of motion artifact.

An in vitro system for studying the relationship between indirect pressure oscillations and direct arterial pressure was developed.

Sudies demonstrated that the air filled system did indicate systolic and diastolic transitions in the oscillometric curve that can be identified and used to indirectly measure the systolic and diastolic simulated arterial pressures.

Studies comparing direct and oscillometric pressure measurements in dogs indicated that oscillometric theory can be used to indirectly measure systolic and diastolic pressure parameters. This project will be terminated and another study initiated to develop a computer algorithm to enhance oscillometric measurement potential in awake animals.

Presentations:

E.C. Walker: Oscillometry: Systolic and Diastolic Pressures in the Dog. 35th ACEMB, 1982.

E.C. Walker: An In Vitro System for Studying the Relationship Between Indirect Pressure Oscillation and Direct Arterial Pressure. 17th. Annual Meeting of AAMI, 1982.

Publication:

1. Walker, E. Oscillometric measurement of penpheral arterial pressure in the laboratory dog. Thesis, 1982; John Hopkins University.

f25

ANNUAL REPORT OF THE
SECTION ON THEORETICAL BIOPHYSICS
OFFICE OF THE DIRECTOR OF INTRAMURAL RESEARCH
NATIONAL HEART, LUNG, & BLOOD INSTITUTE
October 1, 1981 through September 30, 1982

The primary interest of the Section on Theoretical Biophysics is the theory of transport processes in biological systems, with particular reference to problems in cardiovascular, renal, and membrane physiology. The section is concerned both with the formulation of theoretical models and with the development of mathematical and computational methods for their analysis. Currently much of the research in the section centers on the mechanism of urine formation in the mammalian kidney and on theoretical aspects of solute and water transport in epithelial structures.

During the past year, areas of work have included: (1) The thermodynamic and kinetic analysis of transport processes, (2) the qualitative analysis of equations describing kidney models, (4) the development and theoretical analysis of numerical methods, and (5) the computer simulation of renal function.

In a synthesis of earlier work, five theoretical principles that follow from qualitative consideration of renal architecture and tubular permeabilities have been proposed to explain the concentration of urine in the mammalian kidney. These are: (1) The medullary loop of the doubly folded S-shaped configuration of the nephron permits solute supplied by ascending Henle's limb (AHL) to extract water from descending Henle's limb (DHL) and collecting duct (CD). (2) The cortical loop allows the diluted AHL fluid to return to isotonicity with cortical plasma before returning to the medulla. (3) The folded vasa recta and surrounding interstitium (the central core) provide an expansion chamber for the performance of osmotic work and a mixing chamber for salt and urea. This mixing induces passive salt transport out of AHL. (4) Overall, the system acts as a solute cycling multiplier from AHL to vascular core and the osmotically equilibrated DHL and CD. (5) The short-looped nephrons provide urea to drive salt transport out of AHL of long nephrons in the inner medulla (Stephenson).

In further study of multiple solutions and hysteresis phenomena, diffusive dissipation, which is an approximation of vascular washout of solute, has been incorporated into the analytic treatment of the n-stage central core model. The existence of such dissipation seems to be essential for the existence of a stable upper branch of the concentration ratio.

We have also extended our analytic treatment of multisolute systems. We have shown that under idealizing assumptions in a system with nephrons with both short and long loops of Henle the

maximum concentration ratio that can be generated in the inner medulla by the passive mechanism, i.e., salt and urea mixing, is

$$x = \frac{1 + n}{1 + n\, C_{1U}(0)/C_{1M}(0)}$$

where n - 1 is the ratio of short to long nephrons and $C_{1U}(0)/C_{1M}(0)$ is the ratio of urea concentration to total concentration in tubular fluid at the end of the proximal tubule. This work is being extended to include the effects of urine withdrawal and vascular dissipation. These both tend to limit concentration as the ratio of short to long nephrons increases.

Computer simulation of the system shows an optimum ratio of short to long nephrons of somewhere between 3 and 10. This is consistent with the known architecture of the medulla in desert rodents and the hypothesis that these rodents utilize the passive mechanism for concentration in the inner medulla. (Stephenson and Mejia).

Experiments done with a model containing two nephron populations have shown that a passive concentrating mechanism in the inner medulla is enhanced by urea reabsorption from the thick ascending limb of Henle. Uptake by the vasa recta appears to increase concentrating ability while recycling via the descending loops of Henle reduces the ability to concentrate. Conversely, experiments with a two-nephron central core model show that urea recycled into the short loops of Henle enhances concentration.

These computer studies have utilized a previously described, path-following procedure for studying solutions to large sparse systems of nonlinear equations. This method has been extended by R. Mejia during the past year. An interactive program for following solutions for large systems is currently being developed.

In additional work on computational methodology, an interactive solver for small systems of partial differential equations has been developed using the continuation algorithm SCOUT (see H. Jurgens, H-O. Peitgen and D. Saupe, Topological Perturbations in the Numerical Study of Nonlinear Eigenvalue and Bifurcation Problems, Proc. Symposium on Analysis and Computation of Fixed Points, S. M. Robinson, editor, Academic Press (1980)). This solver has been used to test various discretization schemes for the computation of washout curves following arterial injection of a variety of substances such as PAH and radioactive tracers. A backward finite differences scheme in both space and time has been selected as most appropriate.

SCOUT and the symbolic manipulator MACSYMA have been used by R. Mejia to do a first order sensitivity analysis of a 4-tube central core model. The analysis shows that multiple (time) stable states do not exist for small variations of model parameters in models without diffusion and in models where diffusion dominates

convection. It also shows that for moderate diffusion, multiple stable states exist. The general theoretical analysis of this system is discussed below.

In closely correlated collaborative studies, R. Tewarson has carried out computational experiments with a method using the variables and their derivatives to improve the accuracy of integrating the flow equations. He has also developed a seventh order numerical method for integrating the tubal equations and an improved matrix method for iterating the solution of renal models.

The general purpose of this intensive work on numerical methods is the development of efficient methods of solving the renal models. This is essential if the behavior of the models as a function of their parameters, i.e., such as tubular permeabilities, is to be explored in any reasonable time and at any reasonable cost. Such exploration is a first step in the development of parameter estimation schemes that will permit at least some of the microscopic parameters to be determined by comparing the behavior of the models with that of the kidney.

A collaborative effort of Drs. J. Gonzalez-Fernandez and L. Lara (MRB, NIADDKD), Dr. E. Cabib (LBM, NIADDKD), and R. Mejia to study the morphogenesis of the yeast, Saccharomyces cerevisiae has begun. The approach is to incorporate experimental biochemical data into a mathematical formalism of growth. As a first step, a model for the growth of the septum between mother and daughter cells is being developed and implemented numerically. This work offers a new and interesting application of our capability in computer simulation.

Work has continued on the mathematical analysis of a simple central core model of the renal medulla. The model uses a system of differential equations to represent the flow of water and a single solute in a 4- tube flow network, representing the loop of Henle, the collecting duct, and interstitial space. The model includes active transport out of the ascending limb, water extraction from the descending limb, and passive transport of water to and from the collecting duct. An existence theorem is proved for solutions of the model equations that generalizes a previous result obtained for a 3-tube model of the renal medulla. A simplified system of equations is obtained for the limiting case of large water permeabilities, and the simplified system is solved exactly. It develops that, for a given system of boundary conditions, there may be one, two, or three solutions to the simplified system. The solution surfaces have been explored for a variety of parameter values. (Kellogg , Garner, and Stephenson)

A study has been made of the system of ordinary differential equations that describes the flow of water and a solute in a system of parallel flow tubes. The tubes are allowed to exchange water and solute with each other through the tube walls. Several different types of boundary conditions at the ends of the tubes are treated. These boundary conditions are designed to model open-ended tubes,

closed-ended tubes, or connections between two tubes of the system. Some a priori bounds on the solutions of the system are obtained. These bounds have the consequence that the concentration profiles are positive and that, if there is no active transport of solute in the system, the concentration never exceeds one of the specified inlet concentrations. This latter result generalizes an earlier result of Stephenson [Biophysical J 6:539-551, 1966]. The bounds are used in a fixed point argument to obtain existence theorems for solutions of the nonlinear system. (Kellogg and Garner).

Work has started on a local existence theorem for a general multisolute model of the full nephron. The goal of the study is to generalize the above results and include the most general flow network that might be used in renal models.

A project has been initiated on the analysis of the transport of p-aminohippurate and related substances in the mammalian kidney. This project is directed primarily toward the analysis of the kinetics of transport of radionuclides, such as 131-I-Hippuran, that are used in clinical studies. We have now developed a stable numerical scheme for approximating the differential equations that describe the uptake of PAH by proximal tubule cells and its subsequent course through the kidney. The temporal and spatial patterns of concentration predicted by the model are in good agreement with those found experimentally. The next step will be to develop procedures for estimating the parameters of the model from experimental data.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03201-22 STB

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Mathematical Theory of Renal Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. L. Stephenson	Chief, Section on Theoretical Biophysics	OD NHLBI
OTHERS:	R. Mejia	Mathematician	OD NHLBI
	B. Kellogg	Prof. IPST, University of Maryland	
	J. Garner	Prof. Louisiana Tech University, Ruston, LA.	
	R. Kaimal	Visiting Fellow, Fogarty International Center	

COOPERATING UNITS (if any)
Mathematical Research Branch, NIADDKD; IPST, University of Maryland; Louisiana Tech University, Ruston, LA.

LAB/BRANCH

SECTION
Section on Theoretical Biophysics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.7	2.2	.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to develop the general theory of transport and flow processes taking place in the kidney. Aims of current work include (1) Thermodynamic and kinetic analysis of flow processes, (2) the qualitative analysis of equations describing kidney models, (3) the development of analytical solutions of kidney models, and (4) development of a model of the transport of PAH and related substances.

830

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: The primary purpose of this project is to develop the general theory of the transport and flow taking place in the kidney. This includes the thermodynamic and kinetic analysis of flow processes; the development of analytical solutions of kidney models, and the qualitative analysis of equations describing kidney models.

Major findings:

Work has continued on studies of the mechanism of urinary concentration. Diffusive dissipation, which is an approximation of vascular washout of solute, has been incorporated into the analytic treatment of the n-stage central core model. The existence of such dissipation seems to be essential for the existence of a stable upper branch of the concentration ratio. We have also extended our analytic treatment of multisolute systems. We have shown that under idealizing assumptions in a system with nephrons with both short and long loops of Henle the maximum concentration ratio that can be generated in the inner medulla by the passive mechanism is

$$x = \frac{1 + n}{1 + n\, C_{1U}(0)/C_{1M}(0)}$$

where n - 1 is the ratio of short to long nephrons and $C_{1U}(0)/C_{1M}(0)$ is the ratio of urea concentration to total concentration in tubular fluid at the end of the proximal tubule. This work is being extended to include the effects of urine withdrawal and vascular dissipation. These both tend to limit concentration as the ratio of short to long nephrons increases. Computer simulation of the system shows an optimum ratio of short to long nephrons of somewhere between 3 and 10. This is consistent with the known architecture of the medulla in desert rodents and the hypothesis that these rodents utilize the passive mechanism for concentration in the inner medulla. (Stephenson)

Five theoretical principles that follow from qualitative consideration of renal architecture and tubular permeabilities have been proposed to explain the concentration of urine in the mammalian kidney. These are: (1) The medullary loop of the doubly folded S-shaped configuration of the nephron permits solute supplied by ascending Henle's limb (AHL) to extract water from descending Henle's limb (DHL) and collecting duct (CD). (2) The cortical loop allows the diluted AHL fluid to return to isotonicity with cortical plasma before returning to the medulla. (3) The folded vasa recta and surrounding interstitium (the central core) provide an expansion chamber for the performance of osmotic work and a mixing chamber for salt and urea. This mixing induces passive salt transport out of AHL. (4) Overall, the system acts as a solute cycling multiplier from AHL to vascular core and the osmotically equilibrated DHL and CD. (5) The short-looped nephrons provide urea to drive salt transport out of AHL of long nephrons in the inner medulla. This work was presented as part of a symposium on the urinary

concentrating mechanism, organized and chaired by J.L. Stephenson at the Spring, 1982, FASEB meetings.

Work has continued on the mathematical analysis of a simple model of the renal medulla. The model uses a system of differential equations to represent the flow of water and a single solute in a 4-tube flow network, representing the loop of Henle, the collecting duct, and interstitial space. The model includes active transport out of the ascending limb, water extraction from the descending limb, and passive transport of water and solute to and from the collecting duct. An existence theorem is proved for solutions of the model equations that generalizes a previous result obtained for a 3-tube model of the renal medulla. A simplified system of equations is obtained for the limiting case of large water permeabilities, and the simplified system is solved exactly. It develops that, for a given system of boundary conditions, there may be one, two, or three solutions to the simplified system. The solution surfaces have been explored for a variety of parameter values. (Kellogg and Garner)

A study has been made of the system of ordinary differential equations that describes the flow of water and a solute in a system of parallel flow tubes. The tubes are allowed to exchange water and solute with each other through the tube walls. Several different types of boundary conditions at the ends of the tubes are treated. These boundary conditions are designed to model open-ended tubes, closed-ended tubes, or connections between two tubes of the system. Some a priori bounds on the solutions of the system are obtained. These bounds have the consequence that the concentration profiles are positive and that, if there is no active transport of solute in the system, the concentration never exceeds one of the specified inlet concentrations. This latter result generalizes an earlier result of Stephenson [Biophysical J 6:539-551, 1966]. The bounds are used in a fixed point argument to obtain existence theorems for solutions of the nonlinear system. The results have appeared. (Kellogg and Garner)

Work has started on a local existence theorem for a general multisolute model of the full nephron. The goal of the study is to generalize the above results and include the most general flow network that might be used in renal models.

A project has been initiated on the analysis of the transport of p-aminohippurate and related substances in the mammalian kidney. This project is directed primarily toward the analysis of the kinetics of transport of radionuclide such as 131-I-Hippuron that are used in clinical studies. We have now developed a stable numerical scheme for approximating the differential equation that describes the uptake of PAH by the proximal tubule cells and its subsequent course through the kidney. This scheme and its computational results were presented at the FASEB meetings (Fed Proc

41:1240 abstract 5663) and are being written up for publication. (Kaimal, Kellogg, Stephenson)

Proposed course:

One major objective of our research is to understand the cause and the possible physiological significance of the multiple solutions of the kidney models. Toward this end we plan further analysis of both the analytic models and the detailed models requiring numerical solution.

A second objective is to incorporate a more detailed model of proximal tubular transport into the whole kidney model. This is the first step in developing a model of electrolyte transport in the whole kidney. We also plan to develop an approximate analytic model of the whole kidney.

A third objective is to develop procedures for estimating the parameters of the PAH model from images obtained with a scintillation camera following OIH injection.

Publications:

1. Garner, J. B. and Kellogg, R. B. 1982. Diffusion and convection in a family of tubes. J. Math. Anal. and Applications 85:461-472.

2. Stephenson, John L. 1981. Concentrating engines and the kidney. IV. Mass balance in a single stage of a multistage model of the renal medulla. Math Biosci. 55:265-278.

3. Stephenson, John L. 1982. Renal Concentrating Mechanism - Introduction. Fed. Proc. (In press).

4. Stephenson, John L. 1982. Renal Concentrating Mechanism. Fundamental Theoretical Concepts. Fed. Proc. (In press).

5. Stephenson, John L. and Berliner, R. 1982. Renal Concentrating Mechanism - Summary. Fed. Proc. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03202-11 STB

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Computer Simulation of Renal Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. Mejia	Mathematician	OD NHLBI
OTHERS:	J. L. Stephenson	Chief, Section on Theoretical Biophysics	OD NHLBI
	R. Tewarson	Prof. SUNY, Stony Brook, L. I., New York	

COOPERATING UNITS (if any)
SUNY, Stony Brook, L.I., New York, and NIADDKD, Mathematical Research Branch

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Section on Theoretical Biophysics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD. 20205

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to develop a computer simulation of the kidney which describes transport of electrolyte, nonelectrolyte and water in both steady state and transient behavior. Current work is directed toward the development and theoretical analysis of efficient numerical methods of solving the differential-integral equations describing the renal counterflow system and of estimating model parameters.

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PHS-6040
(Rev. 2-81)

Project Description:

Objectives: The purpose of this project is to develop a computer simulation of the mammalian kidney that gives a realistic description of function. This will permit the correlation of micropuncture and macroscopic clearance data with membrane transport characteristics.

Major Findings:

A path-following procedure for studying solutions to large, sparse systems of nonlinear equations has been implemented and described by R. Mejia, An Example of Path-Following in a Subspace, SIAM Conference on Applied Linear Algebra, Raleigh, April 1982. This permits study of model solutions as a function of all model parameters. An interactive program for analyzing the path of solutions for large systems is currently being developed.

A manuscript describing a path-following study of the solution surface of a multinephron model has been prepared to be submitted for publication. It shows that the transition from the non-concentrating kidney to the concentrating (or diluting) kidney lies on a convoluted solution surface. For a given set of model parameters there may be no solution; the solution may be unique, or there may be a multiplicity of solutions.

Experiments done with a model containing two nephron populations have shown that a passive concentrating mechanism in the inner medulla is enhanced by urea reabsorption from the thick ascending limb of Henle. Uptake by the vasa recta appears to increase concentrating ability while recycling via the descending loops of Henle reduces the ability to concentrate. Conversely, experiments with a two-nephron central core model show that urea recycled into the short loops of Henle enhances concentration.

It was previously reported that a multigrid finite differences algorithm had been developed to solve a multinephron model with many solutes. It makes use of the partitioning scheme described by R. Mejia and J. L. Stephenson, Numerical Solution of Multinephron Kidney Equations, J. Computational Phys. 32 (1979) in conjunction with a finite differences scheme described by R. P. Tewarson, On the Use of Simpson's Rule in Renal Models, Math. Biosci. 55 (1981) and a halved mesh spacing for calculation of the global variables. We have now shown that this algorithm permits spurious solutions to the differential equations, although accurate and efficient in solving the difference equations. Alternatives are being considered.

An interactive solver for small systems of partial differential equations has been developed using the continuation algorithm SCOUT (see H. Jurgens, H-O. Peitgen and D. Saupe, Topological Perturbations in the Numerical Study of Nonlinear Eigenvalue

and Bifurcation Problems, Proc. Symposium on Analysis and Computation of Fixed Points, S. M. Robinson, editor, Academic Press (1980)). This solver has been used to test various discretization schemes for the computation of washout curves following arterial injection of a variety of substances such as PAH and radioactive tracers. A backward finite differences scheme in both space and time has been selected as most appropriate.

SCOUT and the symbolic manipulator MACSYMA have been used to do a first order sensitivity analysis of a 4-tube central core model (R. Mejia, Sensitivity Analysis of a Central Core Model of Renal Concentration, Navy MACSYMA Users Mini-Symposium, DTNSRDC, March 1982). The analysis shows that multiple (time) stable states do not exist for small variations of model parameters in models without diffusion and in models where diffusion dominates convection. It also shows that for moderate diffusion, multiple stable states exist.

In closely correlated collaborative studies, R. Tewarson has carried out computational experiments with a method using the variables and their derivatives to improve the accuracy of integrating the flow equations. He has also developed a seventh order numerical method for integrating the tubal equations and an improved matrix method for iterating the solution of renal models.

Proposed Course

(1) Factors affecting urea cycling and excretion will be studied with whole kidney and central core models. (2) Sensitivity analysis of the models should serve to identify key parameters in the concentrating mechanism.

Annual Report of the Pathology Branch
Division of Intramural Research
National Heart, Lung, and Blood Institute
October 1, 1981 through September 30, 1982

Investigations during the above time period as in previous years centered primarily on studies of coronary, valvular, congential, and myocardial heart diseases.

CORONARY HEART DISEASE

The relation of serum total cholesterol and triglyceride levels to the amount and extent of coronary arterial narrowing by atherosclerotic plaques was determined at necropsy in 40 patients with fatal coronary heart disease. The patients were divided into 4 groups based upon the total cholesterol and triglyceride levels and then the amount of cross-sectional area narrowing by atherosclerotic plaques in each of the 4 major coronary arteries was determined by dividing these arteries into 5-mm segments and determining the percent luminal narrowing in each segment. The percents of 5-mm segments narrowed severely correlated significantly with the serum triglyceride levels, but the serum total cholesterol level did not correlate with the percent of 5-mm segments of major coronary arteries severely narrowed.

Numerous epidemiologic and angiographic studies have analyzed the association of serum cholesterol and triglyceride levels with coronary arterial disease. We examined the coronary arteries in a quantitative manner in 43 necropsy patients with types II, III or IV hyperlipoproteinemia or normal lipoprotein patterns. The patients with type IV hyperlipoproteinemia had a higher percentage of 5-mm segments of each of the 4 major coronary arteries narrowed greater than 75% in cross-sectional area than did the patients with type II or III hyperlipoproteinemia who had similar severe degrees of coronary narrowing.

Coronary, as well as other arterial atherosclerotic plaques, frequently contain extravasated erythrocytes, iron and fibrin. The frequency of these 3 factors and their relation to intraluminal thrombus however, has not been determined. We analyzed the presence of extravasated erythrocytes, iron and fibrin within coronary atherosclerotic plaques by dividing each of the 4 major coronary arteries from 57 patients into 2958 5-mm segments and compared the findings to study of 1290 5-mm segments of 103 coronary arteries in 27 control subjects. Intraplaque extravasated erythrocytes were present in 10% of the segments (controls [c] = 1%); in 35% of the arteries (c = 5%), and in 86% of the patients (c = 18%), and in 56% of the patients (c = 14%); intraplaque fibrin was present in 2% of the segments (c = <1%), in 17% of the arteries (c = 3%) and in 49% of the patients (c = 11%). Intraluminal thrombus, present only in the patients with acute myocardial infarction and in none of the controls, occurred in 3% of the segments, in 8% of the arteries and in 26% of the patients. Ingraplaque hemorrhage or extravasated erythrocytes occurred usually in the absence of intraluminal thrombus, and conversely, intraluminal thrombus occurred more frequently without than with underlying plaque hemorrhage. The frequency of intraplaque extravasated erythrocytes, iron and fibrin was proportional to the amount of coronary atherosclerotic plaque present, but intraluminal thrombus was not. Intraplaque iron and fibrin rarely was observed in the absence of extravasated erythrocytes.

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No previous study has compared the extent of coronary artery narrowing to the size of a healed myocardial infarct in patients with clinically recognized or in patients with clinically unrecognized myocardial infarcts. We described clinical and necropsy observations in 61 patients with a healed myocardial infarct, 33 with and 28 without clinical histories of acute myocardial infarction. No significant differences occurred between the 2 groups of patients in mean age, sex or frequency of angina pectoris, chronic congestive heart failure, systemic hypertension, sudden coronary death or fatal acute myocardial infarction. Compared to the patients with clinically recognized acute myocardial infarction, the patients with clinically unrecognized (silent) infarction had a significantly ($p < .05$) higher frequency of diabetes mellitus (43% - vs - 15%), death from non-cardiac causes (39% - vs - 9%), posterior (inferior) wall infarcts (82% - vs - 55%), and smaller infarcts (mean size 7% - vs - 17% of left ventricular wall). The patients with and without clinically recognized infarcts had similar numbers of the 4 major coronary arteries severely (76-100% in cross-sectional area) narrowed (mean 2.8/4.0 - vs - 2.9/4.0 per patient), insignificant differences in similar overall percents of 5-mm segments of the 4 major coronary arteries severely narrowed (43% - vs - 55%), left anterior descending (39% - vs - 33%) and left circumflex (41% - vs - 41%) coronary arteries.

The relation of a healed transmural myocardial infarct size to length of survival after acute myocardial infarction, age at death and amount and extent of coronary arterial narrowing by atherosclerotic plaque was determined in 70 necropsy patients. The mean myocardial infarct size in the 12 patients with and in the 44 without severe narrowing of the left main coronary artery was identical (each = 13%). The percent of 5-mm segments with severe (cross-sectional area narrowing 76-100%) narrowing by atherosclerotic plaques in each patient ranged from 3-93% (mean = 44) and did not correlate with MI size ($r = -0.20$). The entire lengths of the right, left anterior descending and left circumflex coronary arteries in the 56 patients were divided into 5-mm long segments and the amounts of cross-sectional narrowing in each of the resulting 2489 segments was determined by histologic examination. When the 28 patients with a myocardial infarct involving > 10% of the left ventricular wall were compared to those with a myocardial infarct involving $\leq$ 10%, a similar overall percentage of 5-mm segments of coronary artery was severely narrowed (43% - vs - 42%). In addition, a similar percentage of segments were narrowed severely in each of the 3 major epicardial coronary arteries. The healed myocardial infarct size correlated with length of survival after an acute myocardial infarct (in patients with definite histories of an acute myocardial infarct) but not with age at death or with the amount, location or extent of coronary arterial narrowing by atherosclerotic plaques.

Anatomic studies have been made by others of findings in coronary arteries of patients who had undergone coronary angioplasty within hours or days. No late studies however have been available. We examined the major epicardial coronary arteries in 3 patients who had died 80, 90 and 150 days after percutaneous transluminal coronary angioplasty. Each patient had a decrease in the mean transstenotic coronary gradient and a widening of the narrowing at the time of the angioplasty. At necropsy, however, the area of the previous angioplasty in each patient was narrowed 76-95% in cross-sectional area by atherosclerotic plaques and no cracks in plaques and other lesions which may have resulted from the angioplasty procedure were identified histologically in the coronary artery.

Few anatomic studies have been made of coronary arteries in patients with angiographic documentation of coronary arterial spasm. We described clinical and

necropsy findings in 3 patients who had typical Prinzmetal's angina during life and coronary arterial spasm on angiogram. Each died suddenly. Although significant "fixed" coronary narrowing, i.e., that due to atherosclerotic plaques, was appreciated by angiogram in only 1 of the 3 patients, necropsy disclosed severe fixed coronary narrowing in all 3 patients, involving particularly the artery in which spasm had been demonstrated during life. Additionally, examination of each 5-mm long segment of the coronary artery which had been spastic during life (2 patients) disclosed several focally spastic segments at necropsy, indicating that spasm probably persisted after death. Although most previously reported necropsy patients with Prinzmetal's angina had some fixed coronary narrowing, underlying fixed narrowing may be difficult to identify angiographically as demonstrated by our 3 patients.

Tunneling of a major coronary artery into the left ventricular wall has been believed by some investigators to be a cause of myocardial ischemia. We studied at necropsy 2 patients, one aged 34 and the second 48 years, both of whom died because of trauma. Necropsy disclosed that the left main coronary artery in each arose from the right coronary artery and that it burrowed beneath the right ventricular outflow tract within the ventricular septum to appear on the anterior wall of the heart. It then divided into left circumflex and left anterior descending coronary arteries. In each patient, the length of the tunnelled portion of left main coronary artery within left ventricular myocardium or ventricular septum was about 5 cm. At necropsy, none of the coronary arteries was narrowed anatomically and neither patient during life had evidence of cardiac dysfunction. These 2 patients were important in that no cardiac dysfunction occurred and yet the major coronary artery, i.e., the left main, was tunnelled within myocardium for a long distance. Findings in these 2 patients strongly suggest that tunnelling of a major coronary artery has no functional significance.

VALVULAR HEART DISEASE

Until recent years, patients with pure mitral regurgitation were considered to have that lesion primarily on the basis of rheumatic heart disease. We studied operatively excised cardiac valves and then the clinical records in 97 patients over 30 years of age who had undergone mitral valve replacement for pure mitral regurgitation unassociated with aortic valve dysfunction. The etiology of the mitral regurgitation was leaflet prolapse in 60 patients (62%), papillary muscle dysfunction from coronary heart disease in 29 (30%), infective endocarditis on previous normally functioning valves in 5 (5%) and rheumatic heart disease in only 3 (3%). Thus, non-rheumatic conditions caused mitral regurgitation in 94 (97%) of the 97 patients. The history alone usually provided adequate information in the patients with coronary, infective endocarditis and rheumatic etiologies to predict accurately the cause of the mitral regurgitation preoperatively; conversely the lack of a history of one of these 3 conditions strongly suggested mitral valve prolapse to be the cause of the mitral regurgitation. All 60 patients with mitral valve prolapse had increased mitral valve circumferences, leaflet areas and/or products of the circumference times the area. In contrast, only 1 of the 37 patients in whom the mitral regurgitation was due to another cause had increased mitral circumferences, increased areas or the product of the 2.

Rupture of the left ventricular free wall or aneurysm formation has been noted to be a relatively frequent cause of death following mitral valve replacement. Certain clinical and morphologic observations were described in 10 patients who had mitral valve replacement and lacerations of left ventricular free wall midway between the anulus of the mitral valve and the stumps of the left ventricular papillary muscles. In 5 patients, the lacerations led to left ventricular free wall rupture, with immediate hemopericardium in 2 and delayed (2-4 days) rupture in the other 3. Of the other 5 patients, 3 developed aneurysm of the left ventricular free wall, the mouth of which was located midway between mitral anulus and papillary-muscle stumps, the sites of the lacerations observed in the other 7 patients. The remaining 2 patients had midway lacerations which produced neither rupture nor aneurysmal formation. The midway left ventricular lacerations are considered the result of left ventricular incisions made at the time of mitral valve excision. This complication can be prevented by leaving the posterior mitral leaflet and its attached chordae intact or by applying exquisite care during the mitral excision procedure or both. Left ventricular midway rupture may be the most common cause of death early after mitral valve replacement and operatively induced left ventricular lacerations may lead to left ventricular aneurysm late postoperatively.

It is well known that patients with active infective endocarditis often develop valvular regurgitation but it is not appreciated that valvular stenosis may be a consequence of active infective endocarditis. We described 1 patient who developed mitral stenosis purely on the basis of a large vegetation filling much of the previously normal mitral orifice and another patient in whom active infection clearly worsened pre-existing mitral stenosis.

Most ECG studies in patients with aortic valve stenosis have involved living patients in whom the status of the left ventricular myocardium, epicardial coronary arteries, and mitral valve was not precisely known. We examined the 12-lead ECGs recorded within 2 months of death in 50 patients aged 16 to 65 years (mean 48) with peak systolic pressure gradients (PSG) across the aortic valve ranging from 52 to 180 mm Hg (mean 98) and anatomically normal mitral valves. Excluding 4 patients with complete left bundle branch block, 44 (96%) of the other 46 patients had the usual voltage criteria for left ventricular hypertrophy. Measurement of the total 12-lead QRS amplitude, which ranged from 144 to 417 mm (10mm = 1mV) (mean = 257) proved useful for it correlated directly with PSG across the aortic valve and, when the 4 left bundle branch patients were excluded, with the peak left ventricular systolic pressure. The total 12-lead QRS amplitude (mm) was similar in most patients to the left ventricular systolic pressure (mm Hg). Thus, subtraction of the indirect systemic arterial systolic pressure (mm Hg) from the total 12-lead QRS amplitude (mm) provides a reasonable noninvasive prediction of the PSG across the aortic valve in patients with moderate to severe aortic valve stenosis. Additionally, the mean of the total 12-lead QRS amplitude was significantly ($p < 0.05$) greater in the 11 younger ($\leq$ 40 years) than in the 39 older patients (278 mm - vs - 257 mm), in the 14 women than in the 36 men (277mm - vs - 249mm), in the 22 patients with heavier (> 600 gm) hearts (274mm - vs - 244mm), in the 34 patients without compared to the 16 with significant coronary arterial narrowing (270mm - vs - 238mm), and in the 22 patients without compared to the 24 with ECG myocardial damage patterns (269mm - vs - 236mm)

An important project this year was a description of a large group of necropsy patients with the Marfan syndrome. Among 18 necropsy patients with Marfan syndrome,

13 had fusiform aneurysms of the sinus and proximal tubular portions of ascending aorta, severe aortic regurgitation and severe aortic medial degeneration; 3 had dissection involving the entire aorta which was not dilated previously and the aortic media was normal histologically, and 2 patients had isolated mitral regurgitation with grossly and histologically normal aortas. Of the 9 with fusiform ascending aortic aneurysms, 2 ruptured spontaneously; of the remaining 11, 9 died after operations to correct the severe aortic regurgitation. Of the 3 with aortic dissection, 2 had systemic hypertension before the dissection. Although the only cardiovascular manifestation of the Marfan syndrome was mitral regurgitation in 2 patients, 7 of the other 16 patients also had clinical evidence of mitral regurgitation and 11 of the total 18 had anatomic mitral abnormalities including dilated (>11cm) anuli (11 patients), prolapse (7 patients), ruptured chordae tendineae (5 patients) and mitral anular calcium (5 patients).

In a November 1981 article published in the New England Journal of Medicine, St. John Sutton and his associates described a study conducted in the Brompton Hospital, London, to determine the usefulness of routine cardiac catheterization before operative replacement of one or both left sided cardiac valves. A rebuttal paper to that article was written which provided reasons why cardiac catheterization should be performed routinely, except in emergency situations, in patients undergoing valve replacement.

An occasional patient with systemic hypertension has a murmur suggesting the presence of trace or mild aortic regurgitation. We described clinical and morphologic observations in 5 patients who had severe aortic regurgitation from severe systemic hypertension unassociated with aortic dissection and each patient underwent aortic valve replacement. Valve replacement because of aortic regurgitation from systemic hypertension had not been described previously. Why these 5 patients developed severe aortic regurgitation was not determined, however. Nevertheless, systemic hypertension must be added to the list of causes of severe pure aortic regurgitation.

This branch continues to study in detail the hearts of patients who die following replacement of 1 or more cardiac valves with prostheses. Complications of cardiac valve replacement as learned during a 22-year study were summarized during the above time period. Characteristic abnormalities were divided into the following groups : complications to any valve site and to most or all presently available mechanical or bioprosthetic valves; complications limited to a particular valve site irrespective of the type of prosthesis utilized; and complications following combined mitral and aortic valve replacement.

The ultrastructural section of the Pathology Branch has continued the examination of bioprosthetic valves which had been in place for varying periods of time in both human and non-human animals. Described for the first time were intracuspal hematomas characterized by accumulations of blood between the layers of the cusps in porcine aortic valvular bioprostheses. These hematomas were found to be relatively common and their clinical significance varied according to the size of the hematoma and the extent to which it interfered with the mobility of the affected cusps. The frequency of endothelial cells on the surfaces of procine valvular bioprostheses were determined in 43 patients. Endothelial cells were found to grow on the surfaces of bioprostheses only on those present in the mitral position, not in the aortic position. The growth of endothelial cells on the bioprosthetic cusps may serve to increase the structural stability of the cusp. Histologic and

ultrastructural studies were made on bioprosthetic cusps which became torn or perforated after being in place for varying periods of time. A total of 16 porcine valve prostheses implanted in 14 patients were examined. The cuspal tears and perforations unfortunately appear to be more common the longer the prosthetic valve is in place. It is recognized that calcific deposits frequently form on bioprosthetic cusps which have been in place over a long period of time.

MISCELLANEOUS STUDIES

The detailed anatomic structure of parietal pericardium has recieved little attention. The human parietal pericardium in a number of patients was studied in detail, both histologically and ultrastructurally. Several new observations were made on the structures present on its serosal and parietal surfaces.

Human and non-human animals with anthracycline cardiotoxicity have been studied in this Branch for some time. The relation between clinical evidence of anthracycline cardiotoxicity and histologic signs of anthracycline cardiotoxicity was evaluated by reviewing the clinical and morphologic findings in 64 patients studied at necropsy, all of whom had received doxorubicin or daunorubicin chemotherapy during life. Of the 64 patients, 20 (31%) had documented clinical toxicity consisting of impaired left ventricular systolic performance: in 7 (35%) of the 20 patients, histologic signs of toxicity were absent. In the remaining 13 patients, histologic signs of toxicity ranged from mild to severe. Of the 44 (69%) patients without clinical signs of drug toxicity, 21 (48%) had no histologic signs of cardiotoxicity. The results of this study suggest that attempts to monitor cardiotoxicity by serial evaluation of cardiac histology in patients undergoing anthracycline chemotherapy may be seriously limited by the fact that clinical evidence of toxicity may be present without histologic signs of toxicity; likewise, histologic signs of anthracycline toxicity may be present without clinical evidence of toxicity.

The effect of ICRF-187 against anthracycline induced cardiotoxicity was examined in rabbits and beagle dogs, and it caused significant reduction in the incidence and severity of the chronic cardiomyopathy caused in rabbits and dogs by doxorubicin and daunorubicin.

Most metastases to the heart are not visualized by instruments of precision during life. Of 20 necropsy patients with fatal osteogenic sarcoma, 4 had metastases to the heart and because these metastases consisted of bone, they were radiographically visible. The deposits in 3 patients were intracavitary and in 2 caused cardiac dysfunction.

The effects of propranolol and furosemide on minoxidil-induced cardiomyopathy was examined. The acute cardiomyopathy induced in beagle dogs by the administration of minoxidil, a vasodilating antihypertensive agent, is prevented by pretreatment with furosemide but not by pretreatment with propranolol or hydrochlorothiazide.

The mechanisms of hydralyzine induced myocardial necrosis also was examined in rats. Morphologic observations and data on ^{45}Ca uptake indicate that the myocardial necrosis produced by large doses of hydralazine, a vasodilating anti-hypertensive agent, are related to tachycardia, hypoxia and increased flux of calcium into cardiac muscle cells, and prevented by propranolol or verapamil.

ICRF-187's usefulness against pancreatic toxicity of alloxan was investigated. ICRF-187, a bisdioxopiperazine derivative of EDTA, protects against the hyperglycemic and diabetogenic effect of alloxan in mice. The observations made are

consistent with the hypothesis that formation of free radicals is responsible for the pancreatic damage and that ICRF-187 prevents the formation of these radicals.

Studies on selenium and vitamin E deficiency in ducklings have been studied in the Electorn Microscopy Section of this Branch for some time. The interactions between different trace elements were studied in ducklings given diets that were adequate in selenium and vitamin (Se-E) but contained increased amounts of either Ag, Cu, Co, Te, Cd or Zu. These animals developed cardiac lesions which were morphologically similar to those of Se-E deficiency and were prevented by concurrent administration of Se-E.

The Pathology Branch has collaborated with the Pulmonary Branch of the NHLBI in several projects, particularly those concerning patients with fibrotic lung disease. The structure of alveolar epithelial cells in patients with various fibrotic lung disorders was investigated. A total of 31 patients were examined. This study, which was the first detailed description of the morphology of the alveolar epithelial cells, provided a hypothesis to explain the formation of such cells. Two types of cubiodal cells were recognized. They are derived from bronchiolae epithelial cells and that the lattice cells become sources of epithelial renewal in severely damaged alveoli in fibrotic lungs.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03706-01 PA

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Amounts of coronary narrowing in types II, III and IV hyperlipoproteinemia and in known normal lipoprotein patterns

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Henry Scott Cabin, M.D., Clinical Fellow, NHLBI

OTHER: William C. Roberts, M.D. Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Pathology Branch

SECTION

INSTITUTE AND LOCATION

NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Numerous epidemiologic and angiographic studies have analyzed the association of serum cholesterol and triglyceride and hyperlipoproteinemia with coronary arterial disease. Little morphologic information, however, is available on the status of the coronary arteries in patients with hyperlipoproteinemia. In this report, previous studies are reviewed and results of study of the coronary arteries in 43 necropsy patients with types II, III or IV hyperlipoproteinemia or normal lipoprotein patterns are presented.

844

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Publications: Cabin, H S, Roberts, W C: Amounts of coronary narrowing in types II, III and IV hyperlipoproteinemia and in known normal lipoprotein patterns. Cardiovascular Reviews and Reports 3: 699-708, 1982.

845

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 03707-01 PA

PERIOD COVERED
October 1, 1981 to September 30, 1982.

TITLE OF PROJECT (80 characters or less)
Relation of serum total cholesterol and triglyceride levels to the amount and extent of coronary arterial narrowing by atherosclerotic plaques in coronary....

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Henry Scott Cabin, M.D. , Clinical Fellow, Pathology Branch, NHLBI

OTHER: William C. Roberts, M.D., Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)
NONE

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) The amount of cross-sectional area narrowing by atherosclerotic plaques was determined histologically in each 5-mm long segment of the entire lengths of the right, left main, left anterior descending and left circumflex coronary arteries in 40 patients with fatal coronary heart disease and known fasting serum total cholesterol (TC) and triglyceride (Tg) levels. The pathents were divided into four groups based upon the TC and Tg levels: group I = TC $\leq$ 250 mg/dl, Tg $\leq$ 170 mg/dl; group II = TC $\leq$ 250, Tg > 170; group III = TC >250, Tg $\leq$ 170 and group IV = TC > 250, Tg >170. The number of 5-mm segments of coronary artery narrowed severely (76-100% in cross-sectional area) by atherosclerotic plaques in each group was as follows: 172 (34%) of 505 five mm segments from group I; 242 (69%) of 353 segments from group II; 120 (41%) of 295 from group III and 425 (48%) of 884 segments from group IV. The mean percent of 5-mm segments narrowed severely was significantly greater in group II than in group I ($p < .005$) or group III ($p < .01$). Additionally, the mean number of 4 coronary arteries per patient severely narrowed and the number of pathents with severe narrowing of the left main coronary artery were significantly greater in groups II and III than in group I. The percents of 5-mm segments narrowed severely correlated significantly with the serum Tg level ($p < .03$).

Project description: The amount of cross-sectional area narrowing by atherosclerotic plaques was determined histologically in each 5-mm long segment of the entire lengths of the right, left main, left anterior descending and left circumflex coronary arteries in 40 patients with fatal coronary heart disease and known fasting serum total cholesterol (TC) and triglyceride (Tg) levels. The patients were divided into four groups based upon the TC and Tg levels: group I = TC $\leq$ 250 mm/kl, Tg $\leq$ 170 mm/kl; group II = TC $\leq$ 250, Tg > 170; group III = TC > 250, Tg $\leq$ 170 and group IV = TC > 250, Tg > 170. The number of 5-mm segments of coronary artery narrowed severely (76-100% in cross-sectional area) by atherosclerotic plaques in each group was as follows: 172 (34%) of 505 five mm segments from group I; 242 (69%) of 353 segments from group II; 120 (41%) of 295 from group III and 425 (48%) of 884 segments from group IV. The mean percent of 5-mm segments narrowed severely was significantly greater in group II than in group I ($p < .005$) or group III ($p < .01$). Additionally, the mean number of 4 coronary arteries per patient severely narrowed and the number of patients with severe narrowing of the left main coronary artery were significantly greater in groups II and III than in group I. The percents of 5-mm segments narrowed severely correlated significantly with the serum Tg level ($p < .03$). Although it correlated with the number of severely narrowed coronary arteries per patient, the serum TC level did not correlated with the percent of 5-mm segments of coronary artery with severe narrowing.

Publication: Cabin, H.S., Roberts, W.C.: Relation of serum total cholesterol and triglyceride levels to the amount and extent of coronary arterial narrowing by atherosclerotic plaques in coronary heart disease. THE AMERICAN JOURNAL OF MEDICINE in press.

847

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03708-01 PA

PERIOD COVERED
October 1, 1981 to September 30, 1982.

TITLE OF PROJECT (80 characters or less)
Quantitative comparison of extent of coronary narrowing and size of healed myocardial infarct in 33 necropsy patients with clinically recognized and in 28 necropsy patients with clinically unrecognized ("silent") ....

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Henry Scott Cabin, M.D.,Clinical Fellow, Pathology Branch, NHLBI

OTHER: William C. Roberts, M.D., Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) Clinical and necropsy observations are described in 61 patients with a healed transmural myocardial infarction, 33 with and 28 without clinical histories of acute myocardial infarction. No significant differences occurred between the two groups of patients in mean age, sex, or frequency of angina pectoris, chronic congestive heart failure, systemic hypertension, sudden coronary death or fatal acute myocardial infarction. Compared to the patients with clinically recognized acute myocardial infarction, the patients with clinically unrecognized (silent) infarction had a significantly ($p < .05$) higher frequency of diabetes mellitus (43% - vs-15%), death from non-cardiac causes (39% - vs-9%), posterior (inferior) wall infarcts (82% - vs - 55%), and smaller infarcts (mean size 7% - vs-17% of left ventricular wall). The patients with and without clinically-recognized infarcts had similar numbers of the four major coronary arteries severely (76-100% in cross-sectional area) narrowed (mean 2.8 - vs - 2.9/4.0 per patient), insignificant differences in frequencies of severe narrowing of the left main coronary artery (18% - vs - 29%), similar overall percents of 5-mm segments of the 4 major coronary arteries severely narrowed (43-vs-55%), left anterior descending (39%-vs-33%) and left circumflex (41%-vs-41%) coronary arteries.

848

PHS-6040
(Rev. 2-81)

Project description: Clinical and necropsy observations are described in 61 patients with a healed transmural myocardial infarction, 33 with and 28 without clinical histories of acute myocardial infarction. No significant differences occurred between the two groups of patients in mean age, sex, or frequency of angina pectoris, chronic congestive heart failure, systemic hypertension, sudden coronary death or fatal acute myocardial infarction. Compared to the patients with clinically recognized acute myocardial infarction, the patients with clinically unrecognized (silent) infarction had a significantly ($p < .05$) higher frequency of diabetes mellitus (43 - vs - 15%), death from non-cardiac causes (39% - vs - 9%), posterior (inferior) wall infarcts (82%-vs-55%), and smaller infarcts (mean size 7% - vs - 17% of left ventricular wall). The patients with and without clinically-recognized infarcts had similar numbers of the four major coronary arteries severely (76-100% in cross-sectional area) narrowed (mean 2.8 - vs - 2.9/4.0 per patient), insignificant differences in frequencies of severe narrowing of the left main coronary artery (18% - vs - 29%), similar overall percents of 5-mm segments of the 4 major coronary arteries severely narrowed (43 - vs - 55%), left anterior descending (39% - vs - 33%) and left circumflex (41% - vs 41%) coronary arteries.

Publication: Cabin, H.S., Roberts, W.C.: Quantitative comparison of extent of coronary narrowing and size of healed myocardial infarct in 33 necropsy patients with clinically recognized and in 28 necropsy patients with clinically unrecognized ("silent") myocardial infarctions. Circulation, July 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03709-01 PA

PERIOD COVERED
October 1, 1981, to September 30, 1982.

TITLE OF PROJECT (80 characters or less)

Status of the major epicardial coronary arteries 80-150 days after percutaneous transluminal coronary angioplasty: Analysis of 3 necropsy patients.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Bruce F. Waller, Associate Staff Fellow, NHLBI

OTHER: Bruce M. McManus, Senior Staff Fellow, NHLBI
H. Joel Gorfinkel, Department of Medicine, Mt. Carmel Medical Center
Joan C. Kishel, Medical Staff Fellow, NHLBI
Edward C. H. Schmidt, Department of Pathology, Memorial Hospital
Kenneth M. Kent, Georgetown University Medical Center
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

Cardiology Branch; Department of Medicine (Cardiology), Mt. Carmel Medical Center, Columbus, Ohio; Department of Pathology, Memorial Hospital, Easton,MD.

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Certain clinical and necropsy cardiac findings are described in 3 men who had percutaneous transluminal coronary angioplasty (PTCA) of the left anterior descending (LAD) coronary artery 80, 90 and 150 days, respectively, before sudden death. Each patient had a decrease in the mean transstenotic coronary gradient of 17, 38 and 43 mm Hg, respectively, and an angiographic increase in the LAD luminal diameter of 55, 60 and 65%, respectively. At necropsy, the LAD coronary artery in the area of the PTCA in each patient was narrowed 76 to 95% in cross-sectional area by atherosclerotic plaques. No cracks in plaques or other lesions which may have resulted from the PTCA procedure were identified histologically in the LAD coronary artery of any patient.

850

PHS-6040
(Rev. 2-81)

Project description: Certain clinical and necropsy cardiac findings are described in 3 men who had percutaneous transluminal coronary angioplasty (PTCA) of the left anterior descending (LAD) coronary artery 80, 90 and 150 days, respectively, before sudden death. Each patient had a decrease in the mean transstenotic coronary gradient of 17, 38 and 43 mm Hg, respectively, and an angiographic increase in the LAD luminal diameter of 55, 60 and 65%, respectively. At necropsy, the LAD coronary artery in the area of the PTCA in each patient was narrowed 76 to 95% in cross-sectional area by atherosclerotic plaques. No cracks in plaques or other lesions which may have resulted from the PTCA procedure were identified histologyically in the LAD coronary artery of any patient.

Publications: Waller, B.F., McManus, B.M., Gorfinkel, H.J., Kishel, J.C., Schmidt, E.C.H., Kent, K.M., Roberts, W.C.: Status of the major epicardial coronary arteries 80-150 days after percutaneous transluminal coronary angioplasty: Analysis of 3 necropsy patients. Submitted to The American Journal of Cardiology.

851

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03710-01 PA

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Relation of healed transmural myocardial infarct size to length of survival after acute myocardial infarction, age at death, and amount and extent of...

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Henry S. Cabin, Clinical Fellow, Pathology Branch, NHLBI

OTHER: William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The percent of left ventricular wall (including ventricular septum) replaced by scar was determined in 70 necropsy patients with a healed transmural myocardial infarct (MI). The mean MI size in the 12 patients with and in the 44 without severe narrowing of the left main coronary artery was identical (each 13%). The percent of 5-mm segments with severe (cross-sectional area narrowing 76-100%) narrowing by atherosclerotic plaques in each patient ranged from 3-93% (mean 44) and did not correlate with MI size (r=-0.20).

852

PHS-6040
(Rev. 2-81)

Project description: The percent of left ventricular wall (including ventricular septum) replaced by scar was determined in 70 necropsy patients with a healed transmural myocardial infarct (MI). The MI involved from 1-55% (mean 13%) of the left ventricular wall. The ages at death of the patients ranged from 25-82 years (mean 62) and did not significantly correlated with MI size (r=-0.12). Of the 70 patients, 41 (59%) had unequivocal histories of an acute MI: the interval from the MI to death in them ranged from 2-276 months (mean 50) and correlated negatively with MI size (r=-0.32, $p < .05$), and the age at the MI ranged from 26-79 years (mean 58) and did not correlate with MI size (r=-0.05).

The 4 major epicardial coronary arteries were examined quantitatively in 56 patients; the number of coronary arteries with severe narrowing ranged from 1-4 (mean 2.9) and did not correlate with MI size (r=-0.24). The mean MI size in the 12 patients with and in the 44 without severe narrowing of the left main coronary artery was identical (each 13%). The entire lengths of the right, left anterior descending and left circumflex coronary arteries in the 56 patients were divided into 5-mm long segments and the amounts of cross-sectional area narrowing in each of the resulting 2489 segments was determined by histologic examination. The percent of 5-mm segments with severe (cross-sectional area narrowing 76-100%) narrowing by atherosclerotic plaques in each patient ranged from 3-93% (mean 44) and did not correlate with MI size (r=-0.20). When the 28 patients with a MI involving >10% of the left ventricular wall were compared to those with a MI involving $\leq$10%, a similar overall percentage of 5-mm segments of coronary artery was severely narrowed (43%-vs-42%). In addition, a similar percentage of segments were narrowed severely in each of the three major epicardial coronary arteries. Thus, in our necropsy patients with a healed transmural MI, the MI size correlated with length of survival after an acute MI (in patients with definite histories of an acute MI) but not with age at death or with the amount, location or extent of coronary arterial narrowing by atherosclerotic plaques.

Publications: Cabin, H.S., and Roberts, W.C.: Relation of healed transmural myocardial infarct size to length of survival after acute myocardial infarction, age at death, and amount and extent of coronary arterial narrowing by atherosclerotic plaques: analysis of 70 necropsy patients. American Journal of Cardiology, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03711-01 PA

PERIOD COVERED
October 1, 1981, to September 30, 1982.

TITLE OF PROJECT (80 characters or less)
Sudden death in Prinzmetal's angina with coronary spasm documented by angiography. Analysis of 3 necropsy patients.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: William C. Roberts, Chief, Pathology Branch, NHLBI

OTHERS: R. Charles Curry, Jr., Veterans Administration Hospital
Jeffery M. Isner, Tufts-New England Medical Center
Bruce F. Waller, Associate Staff Fellow, NHLBI
Bruce M. McManus, Senior Staff Fellow, NHLBI
Renato Mariani-Constantini, Department of Pathology, James A. Haley VA.
Allan M. Ross, George Washington University

COOPERATING UNITS (if any)
Dept. of Pathology & Medicine(Cardiology), G.W. Univ. Wash.DC; Department of Medicine, University of Florida School of Medicine and the Veterans Administration Hospital, Gainsville FL; Dept. of Pathology, James A. Haley V.A. Hospital, Tampa, FL

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Clinical and necropsy findings are described in 3 patients who had rest angina pectoris, ST segment elevation on electrocardiogram during chest pain, coronary arterial spasm on angiogram and sudden death. Although significant "fixed" coronary narrowing i.e., that due to atherosclerotic plaques, was appreciated by angiogram in only 1 of the 3 patients, necropsy disclosed severe fixed coronary narrowings in all 3 patients, involving particularly the artery in which spasm had been demonstrated during life. Additionally, examination of each 5-mm long segment of the coronary artery which had been spastic during life (2 patients) disclosed several focally spastic segments at necropsy, indicating that spasm persisted after death. Although most previously reported necropsy patients with Prinzmetal's angina had some fixed coronary narrowing, underlying fixed narrowing may be difficult to identify angiographically as demonstrated by our 3 patients.

854

PHS-6040
(Rev. 2-81)

Project description: Clinical and necropsy findings are described in 3 patients who had rest angina pectoris, ST segment elevation on electrocardiogram during chest pain, coronary arterial spasm on angiogram and sudden death. Although significant "fixed" coronary narrowing i.e., that due to atherosclerotic plaques, was appreciated by angiogram in only 1 of the 3 patients, necropsy disclosed severe fixed coronary narrowings in all 3 patients, involving particularly the artery in which spasm had been demonstrated during life. Additionally, examination of each 5-mm long segment of the coronary artery which had been spastic during life (2 patients) disclosed several focally spastic segments at necropsy, indicating that spasm persisted after death. Although most previously reported necropsy patients with Prinzmetal's angina had some fixed coronary narrowing, underlying fixed narrowing may be difficult to identify angiographically as demonstrated by our 3 patients.

Publications: Roberts, W.C., Curry, R.C., Jr., Isner, J.M., Waller, B.F., McManus, B.M., Mariani-Constantini, R., and Ross, A.M.: Sudden death in Prinzmetal's angina with coronary spasm documented by angiography. Analysis of 3 necropsy patients. Am. J. Cardiol. July 1982.

855

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03712-01 PA

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Origin of the left main from the right coronary artery or from the right aortic sinus with intramyocardial tunneling to the left side of the heart via the...

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: William C. Roberts, Chief, Pathology Branch, NHLBI

OTHER: Barry S. Dicicco, Senior Medical Resident, Georgetown Univ. Med. Ctr.
Bruce F. Waller, Associate Staff Fellow, Pathology Branch, NHLBI
Joan C. Kishel, Medical Staff Fellow, Pathology Branch, NHLBI
Stuart L. Dawson, Medical Examiner's Office, Washington, D.C.
John C. Hunsaker III, Medical Examiner's Office, Washington, D.C.
James L. Luke, Medical Examiner's Office, Washington, D.C.

COOPERATING UNITS (if any)
Georgetown Univ. Med. Ctr., Medical Examiner's Office

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Recently we studied the hearts of two patients who died from consequences of knife or bullet wounds. Although during life neither ever had clinical evidence of cardiac dysfunction, at necropsy both had origin of the left main (LM) coronary artery from either the right (R) coronary artery or directly from the right-anterior sinus of Valsalva with intramyocardial coursing within the ventricular septum to the left side of the heart. The course within the septum was 5.0 cm in the 34-year-old man and 4.5 cm in the 48-year-old man. In each patient, the LM exited from the ventricular septum anteriorly and immediately branched into the left anterior descending (LAD) and left circumflex (LC) coronary arteries which thereafter followed their usual courses. The walls of the tunneled LM in each patient were thinner than that of the R, LAD or LC coronary arteries. The course of the R coronary artery was normal. The myocardium was normal. The major coronary arteries in both patients were free of atherosclerotic plaques.

856

Project description: Recently we studied the hearts of two patients who died from consequences of knife or bullet wounds. Although during life neither ever had clinical evidence of cardiac dysfunction, at necropsy both had origin of the left main (LM) coronary artery from either the right (R) coronary artery or directly from the right-anterior sinus of Valsalva with intramyocardial coursing within the ventricular septum to the left side of the heart. The course within the septum was 5.0 cm in the 34-year-old man and 4.5 cm in the 48-year-old man. In each patient, the LM exited from the ventricular septum anteriorly and immediately branched into the left anterior descending (LAD) and left circumflex (LC) coronary arteries which thereafter followed their usual courses. The walls of the tunneled LM in each patient were thinner than that of the R, LAD or LC coronary arteries. The course of the R coronary artery was normal. The myocardium was normal. The major coronary arteries in both patients were free of atherosclerotic plaques.

Publications: Roberts, W.C., Dicicco, B.S., Waller, B.F., Kishel, J.C., Dawson, S.L., Hunsaker, J.C., Luke, J.L.: Origin of the left main from the right coronary artery or from the right aortic sinus with intramyocardial tunneling to the left side of the heart via the ventricular septum. American Heart Journal, August 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03713-01 PA

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Elongate thrombus extending from left ventricular apex to outflow tract: a rare complication of myocardial infarction and diagnosed by 2-dimensional...

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Bruce M. McManus, Senior Staff Fellow, Pathology Branch, NHLBI

OTHER: Samuel D. Goldberg, Department of Cardiology, Shady Grove Adventist Hosp.
Timothy J. Triche, Department of Cardiology, Shady Grove Adventist Hosp.
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

Department of Cardiology, Shady Grove Adventist Hosp.

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Mural thrombi probably develop on the left ventricular (LV) endocardial surface of all patients with transmural LV myocardial infarction (MI). The thrombi are nearly always attached over a broad surface and protrusion into the left ventricular cavity is limited. They may be demonstrable by echocardiography. We recently studied a 54-year-old man who died 6 months after an acute MI and had a most unusual LV thrombus. Two-dimensional echocardiogram postoperatively disclosed a "free floating" mass in the LV cavity. At necropsy, an 11-cm long LV pedunculated thrombus attached by a stalk less than 1 cm broad to the endocardium over a healed transmural MI was present.

858

PHS-6040
(Rev. 2-81)

Project description: Mural thrombi probably develop on the left ventricular (LV) endocardial surface of all patients with transmural LV myocardial infarction (MI). The thrombi are nearly always attached over a broad surface and protrusion into the left ventricular cavity is limited. They may be demonstrable by echocardiography. We recently studied a 54-year-old man who died 6 months after an acute MI and had a most unusual LV thrombus. Dizziness, dyspnea and palpitations 6 weeks before death prompted examination which disclosed a systolic click at the base and electrocardiogram, changes of healed posterior MI. Several days later, he had the sudden appearance of pain, numbness and color changes in both legs, aortogram disclosed total occlusion of the distal abdominal aorta, and the abdominal aorta and common iliac arteries were excised and replaced with a graft. The abdominal aorta and common iliac arteries were totally occluded by clot, presumably embolus from the left ventricle. Two-dimensional echocardiogram postoperatively disclosed a "free floating" mass in the LV cavity. He died of postoperative infectious complications.

At necropsy, an 11-cm long LV pedunculated thrombus attached by a stalk less than 1 cm broad to the endocardium over a healed transmural MI was present. Histologic examination of each 5-mm long segment of each of the 4 major epicardial coronary arteries disclosed that only 3 (3%) of 73 segments were narrowed 76-100% in cross-sectional area by atherosclerotic plaque. This 3# figure represents a much smaller amount of coronary narrowing by atherosclerotic plaque than is usually observed in patients with acute or healed MI, both of which average 35%.

Of over 500 patients studied by us at necropsy with either acute or healed MI, we have observed this type of elongated LV thrombus attached to a narrow base in only 1 previous patient, a 20-year-old man who had traumatic contusion of the LV wall.

Publications:
McManus, B.M., Goldberg, S.D., Triche, T.J., Roberts, W.C.: Elongate thrombus extending from left ventricular apex to outflow tract: a rare complication of myocardial infarction and diagnosed by 2-dimensional echocardiogram. Am Heart J, in press.

859

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03714-01 PA

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Coronary calcium--a clue to angiographic underestimation of coronary luminal narrowing

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Bruce F. Waller, Associate Staff Fellow, Pathology Branch, NHLBI

OTHER: Richard E. Rubin, Division of Cardiology, Georgetown Univ. Med. Ctr.
Francis J. McGrath, Division of Cardiology, Georgetown Univ. Med. Ctr.
Albert A. Del Negro, Division of Cardiology, Georgetown Univ. Med. Ctr.
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)
Division of Cardiology, Georgetown Univ. Med. Ctr.

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Because coronary atherosclerosis is nearly always a diffuse process among patients with symptomatic coronary heart disease (CAD), coronary angiograms in these patients often underestimate the degree of luminal narrowing because sites of maximal narrowing are compared to adjacent sites which also are narrowed but just less extensively. The finding of a calcific deposit adjacent to contrast material in a coronary angiogram, however, is a clue that the luminagram does not indicate the original lumen and that the angiogram (or angiographer) is underestimating the actual amount of narrowing because the calcific deposits are virtually always within atherosclerotic plaques which are located within the intima.

860

PHS-6040
(Rev. 2-81)

Project description: Because coronary atherosclerosis is nearly always a diffuse process among patients with symptomatic coronary heart disease (CAD), coronary angiograms in these patients often underestimate the degree of luminal narrowing because sites of maximal narrowing are compared to adjacent sites which also are narrowed but just less extensively. Usually no clues are available during life in patients with symptomatic CAD to indicate the proportion of the original coronary lumen--i.e., the area enclosed by the internal elastic membrane, occupied by atherosclerotic plaque because a coronary angiogram is a luminagram which only indicates residual lumen and not necessarily original lumen. The finding of a calcific deposit adjacent to contrast material in a coronary angiogram, however, is a clue that the luminagram does not indicate the original lumen and that the angiogram (or angiographer) is underestimating the actual amount of narrowing because the calcific deposits are virtually always within atherosclerotic plaques which are located within the intima. We recently studied a 65-year-old man with severe angina pectoris in whom the angiogram disclosed severe (>75% diameter reduction) narrowing of the left anterior descending and left circumflex coronary arteries but <50% narrowing of the right coronary artery (RCA). A linear calcific deposit in the RCA, however, adjacent to the luminagram indicated that the amount of narrowing in this artery was considerably underestimated and, consequently, three aortocoronary conduits were inserted rather than two as would have been done had not this "calcific clue" been detected.

Publications: Waller, B.F., Rubin, R.E., McGrath, F.J., Del Negro, A.A., and Roberts, W.C.: Coronary calcium--a clue to angiographic underestimation of coronary luminal narrowing, Am Heart J 103(6):1071, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03715-01 PA

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Extravasated erythrocytes, iron and fibrin in atherosclerotic plaques of coronary arteries in fatal coronary heart disease and their relation to ...

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Renu Virmani, Department of Pathology, Vanderbilt University Sch. of Med.

OTHER: William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

Department of Pathology, Vanderbilt University School of Medicine

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The presence of extravasated erythrocytes (EE), iron (I) and fibrin (F) within coronary atherosclerotic plaques and their relation to intraluminal coronary thrombus was determined in 2958 five-mm segments of 224 major epicardial coronary arteries in 27 control subjects. Intraplaque EE were present in 10% of the segments (controls [c] = 1%, in 35% of the arteries (c = 5%), and in 86% of the patients (c = 18%); I was present in 4% of the segments (c = < 1%), in 14% of the arteries (c = 4%), and in 56% of the patients (c = 14%); intraplaque F was present in 2% of the segments (c = < 1%), in 17% of the arteries (c = 3%) and in 49% of the patients (c = 11%).

f62

PHS-6040
(Rev. 2-81)

Project description: The presence of extravasated erythrocytes (EE), iron (I) and fibrin (F) within coronary atherosclerotic plaques and their relation to intraluminal coronary thrombus was determined in 2958 five-mm segments of 224 major epicardial coronary arteries in 57 patients with fatal coronary heart disease and in 1290 five-mm segments of 103 coronary arteries in 27 control subjects. Intraplaque EE were present in 10% of the segments (controls [c] = 1%, in 35% of the arteries (c = 5%), and in 86% of the patients (c = 18%); I was present in 4% of the segments (c = <1%), in 14% of the arteries (c = 4%), and in 56% of the patients (c = 14%); intraplaque F was present in 2% of the segments (c = <1%), in 17% of the arteries (c = 3%) and in 49% of the patients (c = 11%). Intraluminal thrombus, present only in the patients with acute myocardial infarction and in none of the controls, occurred in 3% of the segments, in 8% of the arteries and in 26% of the patients. Intraplaque hemorrhage or EE occurred usually in the absence of intraluminal thrombus, and, conversely, intraluminal thrombus occurred more frequently without than with underlying plaque hemorrhage. The frequency of intraplaque EE, I and F was proportional to the amount of coronary atherosclerotic plaque present, but intraluminal thrombus was not. Intraplaque I and F rarely was observed in the absence of EE.

Publications: Virmani, R., and Roberts, W.C.: Extravasated erythrocytes, iron and fibrin in atherosclerotic plaques of coronary arteries in fatal coronary heart disease and their relation to luminal thrombus: frequency and significance in 57 necropsy patients and in 2958 five-mm segments of 224 major epicardial coronary arteries. Submitted to Arteriosclerosis. A Journal of Vascular Biology and Disease.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03716-01 PA

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Exercise and Sudden Death--Part I and II

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Bruce M. McManus, Senior Staff Fellow, Pathology Branch, NHLBI

OTHER: Bruce F. Waller, Associate Staff Fellow, Pathology Branch, NHLBI
Thomas B. Graboys, Assist. Prof. Med., Brigham Hospital
Jere H. Mitchell, Prof. Internal Med. & Physiology, Harry Moss Heart Ctr.
Robert J. Siegel, Senior Staff Fellow, Pathology Branch, NHLBI
Henry S. Miller, Jr., Prof. of Med. & Cardiology, Bowman Gray Sch. of Med.
Victor F. Froelicher, Assoc. Prof. of Med., Univ. of Calif., San Diego
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)
Brigham Hospital, Harry Moss Heart Ctr., Bowman Gray Sch. of Med., Univ. of Calif., San Diego

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This is a 145 page article which summarizes a number of physiologic and morphologic observations in patients who have died suddenly or are likely candidates for sudden death.

864

PHS-6040
(Rev. 2-81)

Project description: This is a 145 page article which summarizes a number of physiologic and morphologic observations in patients who have died suddenly or are likely candidates for sudden death.

Publications: McManus, B.M., Waller, B.F., Graboys, T.B., Mitchell, J.H., Siegel, R.J., Miller, H.S., Froelicher, V.J., Roberts, W.C.: Exercise and Sudden Death--Part I and Part II. Current Problems in CARDIOLOGY, Volume 6, No. 9 & 10, December 1981-January 1982.

865

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03717-01 PA

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Sudden death while playing professional football

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: William C. Roberts, Chief, Pathology Branch, NHLBI

OTHER: Barry J. Maron, Cardiology Branch, NHLBI

COOPERATING UNITS (if any)

Cardiology Branch, NHLBI

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A recent study of 29 competitive athletes aged 13 to 31 years dying suddenly disclosed that 28 of them died from cardiovascular conditions, the most common being hypertrophic cardiomyopathy. Atherosclerotic coronary heart disease was the cause of death in three. Recently we studied the heart in a young man who died while walking back to the huddle after having run a pass pattern in a professional football game.

866

PHS-6040
(Rev. 2-81)

Project description: A recent study of 29 competitive athletes aged 13 to 31 years dying suddenly disclosed that 28 of them died from cardiovascular conditions, the most common being hypertrophic cardiomyopathy. Atherosclerotic coronary heart disease was the cause of death in three. Recently we studied the heart in a young man who died while walking back to the huddle after having a run a pass pattern in a professional football game. He had been well until September 5, 1971 (six weeks before death), when at about 10 p.m. he developed intense upper abdominal pain, which also was associated with "aching" in his shoulders, brief syncope, severe sweating, and bradycardia. Several hours later he was hospitalized by the team surgeon. On admission, the pain was still present, blood pressure was 140/90 mm Hg, and heart rate was 90 beats per minute (bpm) and regular. No precordial murmurs were present. Neither the liver not the spleen was palpable. The chest roentgenogram was normal.

By the next day, the pain had nearly disappeared without the patient's having received any medicines, and on September 7 he was discharged. Because of fever at home, he was rehospitalized on September 9. The serum lactic dehydrogenase was elevated. The results of intravenous pyelogram, selective splenic and renal angiograms, and studies of renal function (to rule out splenic rupture) were normal. The pain did not recur and he was discharged on September 12, 1971. He resumed football practice and was apparently asymptomatic until October 28, 1971, when during the course of a professional football game, he suddenly died. At necropsy, the heart weighed 410 gm and its posterolateral wall was the site of a large transmural healing infarct, consistent with a 6-week duration. The right, left anterior descending, and left circumflex coronary arteries were diffusely atherosclerotic and severely narrowed by atherosclerotic plaques, many of which contained extravasated erythrocytes. A small thrombus with underlying plaque hemorrhage was present in the right coronary artery. Both the liver (2200 gm) and spleen (640 gm) were enlarged.

Reexamination of the clinical features after death in this patient make it clear that he had an acute myocardial infarction 6 weeks before death, and that the "left upper quadrant" pain almost surely was pain of acute myocardial infarction. Although no electrocardiogram or family historical information was recorded in his last 6 weeks of life, interview and examination on July 1, 1971 (less than 4 months before death--at the beginning of football practice) had disclosed that his total serum cholesterol was elevated (350 mg/dl), that his electrocardiogram was within normal limits, and that many members of his family had documented type II hyperlipoproteinemia with or without symptomatic or fatal coronary heart disease.

The unusual anatomic feature about the epicardial coronary arteries in this patient was the extensiveness of the hemorrhages into the atherosclerotic plaques. Tackling and blocking, such as the present patient did, almost surely caused contact of the anterior surface of the heart with the underlying sternum. As long as the coronary arteries are normal or near normal, as is presumably the usual situation in professional football players, the arteries absorb these "blows" without consequence since they are freely pliable. When these arteries are heavily atherosclerotic, as in the present patient, their pliability is lost and then contact of the surface of the heart with the underlying sternum logically might result in "cracking" of the atherosclerotic plaques, allowing hemorrhage

into them in the manner shown in Fig. 4. The crack in the plaque in turn may lead to intraluminal thrombosis. Additionally, "jarring" without actual contact of heavily atherosclerotic coronary arteries, such as the left circumflex or posterior portion of the right, might also produce cracks in plaques with resulting plaque hemorrhage.

Publications: Roberts, W.C., and Maron, B.J.: Sudden death while playing professional football. Am Heart J 102(6):1061, 1982

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03718-01 PA

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Etiology of clinically isolated, severe, chronic, pure mitral regurgitation: analysis of 97 patients over 30 years of age having mitral valve replacement

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Bruce F. Waller, Associate Staff Fellow, Pathology Branch, NHLBI

OTHER: Andrew G. Morrow, Surgery Branch, NHLBI
Barry J. Maron, Cardiology Department, NHLBI
Albert A. Del Negro, Georgetown University Medical Center
Kenneth M. Kent, Cardiology Department, NHLBI
Francis J. McGrath, Georgetown University Medical Center
Robert B. Wallace, Georgetown University Medical Center
Charles L. McIntosh, Surgery Branch, NHLBI
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

Surgery and Cardiology Branches, NHLBI

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The etiology of clinically isolated, severe, pure mitral regurgitation was determined in 97 patients over 30 years of age by examination of the operatively excised mitral valves. None had any degree of mitral stenosis or a dysfunctioning aortic valve as determined by preoperative catheterization. The etiology of the mitral regurgitaiton was leaflet prolapse in 60 patients (62%), papillary muscle dysfunction from coronary heart disease in 29 (30%), infective endocarditis on previously normally functioning valves in 5 (5%), and rheumatic heart disease in 3 (3%).

869

Project description: The etiology of clinically isolated, severe, pure mitral regurgitation was determined in 97 patients over 30 years of age by examination of the operatively excised mitral valves. None had any degree of mitral stenosis or a dysfunctioning aortic valve as determined by preoperative catheterization. The etiology of the mitral regurgitation was leaflet prolapse in 60 patients (62%), papillary muscle dysfunction from coronary heart disease in 29 (30%), infective endocarditis on previously normally functioning valves in 5 (5%), and rheumatic heart disease in 3 (3%). Thus, nonrheumatic conditions caused the mitral regurgitation in 94 (97%) of the 97 patients. The history alone (previous acute myocardial infarction, rheumatic fever, active infective endocarditis) usually provided adequate information in the patients with coronary, infective endocarditis and rheumatic etiologies to predict accurately the cause of the mitral regurgitation preoperatively; conversely, the lack of a history of one of these other 3 conditions strongly suggested mitral valve prolapse as the cause of the mitral regurgitation preoperatively. All 60 patients with mitral valve prolapse had increased mitral valve circumferences, leaflet areas, and/or products of the circumference times the area. In contrast, only 1 of the 37 patients in whom the mitral regurgitation was due to another cause had increased mitral circumference, increased area, or the product of the two. A systolic click was not heard preoperatively in any of the 97 patients including the 60 with mitral valve prolapse.

Publications: Waller, B.F., Morrow, A.G., Maron, B.J., Del Negro, A.A., Kent, K.M., McGrath, F.J., Wallace, R.B., McIntosh, C.L., Roberts, W.C.: Etiology of clinically isolated, severe, chronic, pure mitral regurgitation: analysis of 97 patients over 30 years of age having mitral valve replacement. Am Heart J, in press

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03719-01 PA

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
The Marfan Syndrome

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: William C. Roberts, Chief, Pathology Branch, NHLBI

OTHER: Howard S. Honig, Cardiology Fellow, Georgetown University Medical Center

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Among 18 necropsy patients aged 15-52 years (mean 34) with the Marfan syndrome, 13 had fusiform aneurysms of the sinus and proximal tubular portions of ascending aorta, severe aortic regurgitation (AR) and severe aortic medial degeneration; three had dissection involving the entire aorta which was not dilated previously and the aortic media was normal histologically, and two patients had isolated mitral regurgitation with grossly and histologically normal aortas. Of the nine with fusiform ascending aortic aneurysms, two ruptured spontaneously; of the remaining 11, nine died after operations to correct the severe AR. Of the three with aortic dissection, two had systemic hypertension before the dissection. Although the only cardiovascular manifestation of the Marfan syndrome was MR in two patients, seven of the other 16 patients also had clinical evidence of MR and 11 of the total 18 had anatomic mitral abnormalities including dilated (>11 cm) anuli (11 patients), prolapse (7 patients), ruptured chordae tendineae (5 patients) and mitral anular calcium (5 patients).

871

PHS-6040
(Rev. 2-81)

Project description: Among 18 necropsy patients aged 15-52 years (mean 34) with the Marfan syndrome, 13 had fusiform aneurysms of the sinus and proximal tubular portions of ascending aorta, severe aortic regurgitation (AR) and severe aortic medial degeneration; three had dissection involving the entire aorta which was not dilated previously and the aortic media was normal histologically, and two patients had isolated mitral regurgitation with grossly and histologically normal aortas. Of the nine with fusiform ascending aortic aneurysms, two ruptured spontaneously; of the remaining 11, nine died after operations to correct the severe AR. Of the three with aortic dissection, two had systemic hypertension before the dissection. Although the only cardiovascular manifestation of the Marfan syndrome was MR in two patients, seven of the other 16 patients also had clinical evidence of MR and 11 of the total 18 had anatomic mitral abnormalities including dilated (>11 cm) anuli (11 patients), prolapse (7 patients), ruptured chordae tendineae (5 patients) and mitral anular calcium (5 patients).

Analysis of 151 previously reported necropsy patients with the Marfan syndrome disclosed that 53 (35%) had fusiform ascending aortic aneurysms, 57 (38%) had aortic dissection, 33 (22%) had isolated or predominant MR, and 8 (5%) had miscellanous conditions (mainly normal hearts and ascending aortas). The mean age at death in the patients with either fusiform ascending aortic aneurysm or dissection was similar (28 years) and the mean age of the patients with MR was much younger (12 years). Nearly three fourths of the patients with MR were aged 15 years or younger whereas the percentage of young patients with aneurysm or dissection was much less (15% and 7%, respectively). As with our patients, the occurrence of dissection was infrequent in the previously reported patients with fusiform ascending aortic aneurysm. Dissection appeared to involve aortas which previously were of normal size or only mildly dilated and the degree of "cystic medial necrosis" in them appeared to have been relatively mild. Thus, the aneurysmally dilated ascending aorta, the most common cuase of AR in these patients, is generally not a candidate for dissection but it is prone to complete rupture. And finally, mitral anular dilation with or without leaflet prolapse appears to be the major cause of MR in patients with the Marfan syndrome.

Publications: Roberts, W.C. and Honig, H.S.: The spectrum of cardiovascular disease in the Marfan syndrome: a clinico-morphologic study of 18 necropsy patients and comparison to 151 previously reported necropsy patients. American Heart Journal 104:115-135, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03720-01 PA

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Electrocardiographic observations in severe aortic valve stenosis: correlative necropsy study to clinical, hemodynamic, and ECG variables demonstrating relation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Robert J. Siegel, Senior Staff Fellow, Pathology Branch, NHLBI

OTHER: William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We examined the 12-lead ECG recorded within 2 months of death in 50 patients aged 16 to 65 years (mean 48) with peak systolic pressure gradients (PSPG) across the aortic valve ranging from 52 to 180 mm Hg (mean 98) and anatomically normal mitral valves. Measurement of the total 12-lead QRS amplitude, which ranged from 144 to 417 mm (10mm=1mV), (mean 257) proved useful for it correlated directly with PSPG across the aortic valve and, when the four LBBB patients were excluded, with the peak LV systolic pressure. The total 12-lead QRS amplitude (mm) was similar in most patients to the LV systolic pressure (mm Hg). Thus, subtraction of the indirect systemic arterial systolic pressure (mm Hg) from the total 12-lead QRS amplitude (mm) provides a reasonable noninvasive prediction of the PSPG across the aortic valve in patients with moderate to severe AS.

PHS-6040
(Rev. 2-81)

Project description: Most ECG studies in patients with aortic valve stenosis (AS) have involved living patients in whom the status of the left ventricular (LV) myocardium, epicardial coronary arteries, and mitral valve was not precisely known. We examined the 12-lead ECG recorded within 2 months of death in 50 patients aged 16 to 65 years (mean 48) with peak systolic pressure gradients (PSPG) across the aortic valve ranging from 52 to 180 mm Hg (mean 98) and anatomically normal mitral valves. Excluding four patients with complete left bundle branch block (LBBB), 44 (96%) of the other 46 patients had the usual voltage criteria for LV hypertrophy (LVH). Measurement of the total 12-lead QRS amplitude, which ranged from 144 to 417 mm (10 mm = 1 mV), (mean 257) proved useful for it correlated directly with PSPG across the aortic valve and, when the four LBBB patients were excluded, with the peak LV systolic pressure. The total 12-lead QRS amplitude (mm) was similar in most patients to the LV systolic pressure (mm Hg). Thus, subtraction of the indirect systemic arterial systolic pressure (mm Hg) from the total 12-lead QRS amplitude (mm) provides a reasonable noninvasive prediction of the PSPG across the aortic valve in patients with moderate to severe AS. Additionally, the mean of the total 12-lead QRS amplitude was significantly ($p < 0.05$) greater in the 11 younger ($\leq$40 years) than in the 39 older patients (278 mm vs 257 mm), in the 14 women than in the 36 men (277 mm vs 249 mm), in the 22 patients with heavier (>600 gm) hearts (274 mm vs 244 mm), in the 34 patients without compared to the 16 with significant coronary arterial narrowing (270 mm vs 238 mm), and in the 22 patients without compared to the 24 with ECG myocardial damage patterns (269 mm vs 236 mm).

Publications: Siegel, R.J., Roberts, W.C.: Electrocardiographic observations in severe aortic valve stenosis: correlative necropsy study to clinical, hemodynamic, and ECG variables demonstrating relation of 12-lead QRS amplitude to peak systolic transaortic pressure gradient. Am Heart J 103:210, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03721-01 PA

PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Severe aortic regurgitation secondary to systemic hypertension (without aortic dissection)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Bruce F. Waller, Associate Staff Fellow, Pathology Branch, NHLBI

OTHER: William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Pathology Branch

SECTION

INSTITUTE AND LOCATION

NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Clinical and morphologic observations are described in five patients who had severe aortic regurgitation (AR) from severe systemic hypertension unassociated with aortic dissection and each patient underwent aortic valve replacement. Although AR of minimal or mild degree is well recognized to occur in patients with systemic hypertension, severe degrees of AR in such patients is rare and, aortic-valve replacement in such patients has not been reported from other centers. Why our five patients developed such severe AR, however, was not determined. Although a rare cause, systemic hypertension, nevertheless, must be added to the list of causes of severe pure AR.

875

Project description: Clinical and morphologic observations are described in five patients who had severe aortic regurgitation (AR) from severe systemic hypertension unassociated with aortic dissection and each patient underwent aortic valve replacement. Although AR of minimal or mild degree is well recognized to occur in patients with systemic hypertension, severe degrees of AR in such patients is rare and, aortic-valve replacement in such patients has not been reported from other centers. Why our five patients developed such severe AR, however, was not determined. Although a rare cause, systemic hypertension, nevertheless, must be added to the list of causes of severe pure AR.

Publications: Waller, B.F., and Roberts, W.C.: Cardiovascular Reviews and Reports, September 1982. Severe aortic regurgitation secondary to systemic hypertension (without aortic dissection).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03722-01 PA

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Mitral valve stenosis produced by or worsened by active bacterial endocarditis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Bruce F. Waller, Associate Staff Fellow, Pathology Branch, NHLBI

OTHER: Bruce M. McManus, Senior Staff Fellow, Pathology Branch, NHLBI
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch, NHLBI

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Regurgitation is the usual complication of active infective endocarditis involving a cardiac valve. The development of valve stenosis entirely from infective endocarditis, of course, is extremely rare and worsening of previously existing valve stenosis by infective endocarditis rarely has been documented. In this report we describe one patient who appeared to have mitral stenosis (MS) purely on the basis of a large vegetation filling much of the previously normal mitral orifice and another patient in whom active infection clearly worsened preexisting MS.

877

Project description: Regurgitation is the usual complication of active infective endocarditis involving a cardiac valve. The development of valve stenosis entirely from infective endocarditis, of course, is extremely rare and worsening of previously existing valve stenosis by infective endocarditis rarely has been documented. In this report we describe one patient who appeared to have mitral stenosis (MS) purely on the basis of a large vegetation filling much of the previously normal mitral orifice and another patient in whom active infection clearly worsened preexisting MS.

The infecting bacterium in both patients was Staphylococcus aureus. Patient #1, a 71-year-old man, initially developed a murmur (grade 3/6) consistent with mitral regurgitation during his last two months of life while febrile. He died from an intracerebral bleed. Patient #2, a 37-year-old man who had had acute rheumatic fever as a child and a grade 1/6 apical diastolic rumble with a loud opening snap for a least three years, increased the intensity of the rumble to grade 3/6 and lost the opening snap when evidence of active infective endocarditis appeared. Cardiac catheterization during the infection disclosed a mean diastolic gradient of 26 mm Hg between pulmonary artery wedge position and left ventricle. The mitral valve was successfully replaced.

Although hemodynamic confirmation is lacking, the finding of a large vegetation filling the mitral orifice of an otherwise normal valve in our patient #1 strongly suggests that mitral stenosis (MS) can be produced by active infective endocarditis alone. Roberts and associates previously reported the occurrence of MS from infective endocarditis alone due to Group B hemolytic Streptococcus in a 21-year-old necropsy woman and Davies and associates later described a 38-year-old man in whom both the mitral and aortic orifices at operation appeared to have been obstructed by vegetations alone (Group C hemolytic Streptococcus).

The occurrence of active infective endocarditis on a previously stenotic mitral valve, of course, may worsen the degree of stenosis although the degree of worsening has never been documented hemodynamically. In our patient #2 and in three previously reported patients, the preexisting MS was clearly worsened by the presence of vegetations. Of these four patients, the infecting bacterium in three was Staphylococcus aureus and in one, the blood cultures and cultures of the operatively-excised vegetations were negative. The excised mitral valves in the three previously reported patients were similar in appearance to that in our patient #2.

Thus, active infective endocarditis can by itself produce MS, and active infective endocarditis may worsen preexisting MS.

Publications: Waller, B.F., McManus, B.M., Roberts, W.C.: Mitral valve stenosis produced by or worsened by active bacterial endocarditis. Chest, July 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03723-01 PA

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Left ventricular incision midway between the mitral anulus and the stumps of the papillary muscles during mitral valve excision with or without rupture or...

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: William C. Roberts, Chief, Pathology Branch, NHLBI

OTHER: Jeffrey M. Isner, Department of Cardiology, Tufts University Med. Ctr.
Renu Virmani, Department of Pathology, Vanderbilt University Med. Ctr.

COOPERATING UNITS (if any)
Department of Cardiology, Tufts University Medical Center
Department of Pathology, Vanderbilt University Medical Center

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Certain clinical and morphologic observations are described in 10 patients who had mitral replacement (MVR) and lacerations of the left ventricular (LV) free wall midway between the anulus of the mitral valve and the stumps of the LV papillary muscles. In 5 patients, the lacerations led to LV free wall rupture, with immediate hemopericardium in 2 delayed (2-4 days) rupture in the other 3. Of the other 5 patients, 3 developed aneurysm of the LV free wall, the mouth of which was located midway between mitral anulus and papillary-muscle stumps, the sites of the lacerations observed in the other 7 patients. The remaining 2 patients had midway lacerations which produced neither rupture nor aneurysmal formation. The midway LV lacerations are considered the result of LV incisions made at the time of mitral valve excision.

879

Project description: Certain clinical and morphologic observations are described in 10 patients who had mitral valve replacement (MVR) and lacerations of the left ventricular (LV) free wall midway between the anulus of the mitral valve and the stumps of the LV papillary muscles. In 5 patients, the lacerations led to LV free wall rupture, with immediate hemopericardium in 2 and delayed (2-4 days) rupture in the other 3. Of the other 5 patients, 3 developed aneurysm of the LV free wall, the mouth of which was located midway between mitral anulus and papillary-muscle stumps, the sites of the lacerations observed in the other 7 patients. The remaining 2 patients had midway lacerations which produced neither rupture nor aneurysmal formation. The midway LV lacerations are considered the result of LV incisions made at the time of mitral valve excision, generally in a setting where the left-sided cardiac chambers were only mildly dilated or normal and the tips of the blades of the scissors may have been inadequately visualized during mitral excision. This complication can be prevented by leaving the posterior mitral leaflet and its attached chordae intact or by applying exquisite care during the mitral excision procedure or both. LV midway rupture may be the most common cause of death early after MVR and operatively induced LV lacerations may lead to LV aneurysm late postoperatively.

Publications: Roberts, W.C., Isner, J.M., and Virmani, R.: Left ventricular incision midway between the mitral anulus and the stumps of the papillary muscles during mitral valve excision with or without rupture or aneurysmal formation: Analysis of 10 necropsy patients. American Heart Journal, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03724-01 PA

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Aortic valve stenosis and left ventricular apical aneurysm and/or rutpure

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: William C. Roberts, Chief, Pathology Branch, NHLBI

OTHER: Ernest N. Arnett, Department of Cardiology, Franklin Square Hospital
Seena C. Aisner, Department of Pathology, Franklin Square Hospital
Paul Techlenberg, Franklin Square Hospital

COOPERATING UNITS (if any)
Departments of Cardiology and Pathology, Franklin Square Hospital, Baltimore, Maryland

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

When acute myocardial infarction (MI) occurs in patients with systemic hypertension, the systemic arterial and left ventricular (LV) pressure generally returns to or toward normal if the MI was fairly large. When acute MI occurs in patients with significant aortic valve stenosis (AS), however, the LV systolic pressure remains elevated and the continuation of this pressure elevation increases the likelihood of LV rupture or aneurysmal formation, particularly when the MI involves the LV apical wall which normally is several times thinner than the LV basal wall. Such was the case in a 67-year-old man, who died of progressive congestive heart failure after healing of more than 1 (by history) acute MI in the previous 5 years. At necropsy, he had a severely stenotic congenitally bicuspid aortic valve and a large apical aneurysm at the site of a healed LV MI.

881

Project description: When acute myocardial infarction (MI) occurs in patients with systemic hypertension, the systemic arterial and left ventricular (LV) pressure generally returns to or toward normal if the MI was fairly large. When acute MI occurs in patients with significant aortic valve stenosis (AS), however, the LV systolic pressure remains elevated and the continuation of this pressure elevation increases the likelihood of LV rupture or aneurysmal formation, particularly when the MI involves the LV apical wall which normally is several times thinner than the LV basal wall. Such was the case in a 67-year-old man, who died of progressive congestive heart failure after healing of more than 1 (by history) acute MI in the previous 5 years. At necropsy, he had a severely stenotic congenitally bicuspid aortic valve and a large apical aneurysm at the site of a healed LV MI. The heart weighed 630 grams. Both the left anterior descending and left circumflex coronary arteries were narrowed 76-100% in cross-sectional area by atherosclerotic plaques.

The occurrence of LV free wall rupture and/or aneurysmal formation has not been reported previously in a patient with severe AS. This fact is surprising in view of the elderly age of many patients with AS.

Publications: Roberts, W.C., Arnett, E.N., Aisner, S.C., and Techlenberg, P.: Aortic valve stenosis and left ventricular apical aneurysm and/or rupture. Real or potential complications of persistent left ventricular systolic hypertension after acute myocardial infarction. Am Heart J, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03725-01 PA

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Sounding Board: Reasons for cardiac catheterization before cardiac-valve replacement

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: William C. Roberts, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In a November 1981 article published in the New England Journal of Medicine, St. John Sutton and his associates described a study conducted in the Brompton Hospital, London, to determine the usefulness of routine cardiac catheterization before operative replacement of one or both left-side cardiac valves. The present paper was a rebuttal to that article providing reasons why cardiac catheterization should be performed routinely except in emergency situations in patients undergoing valve replacement.

883

PHS-6040
(Rev. 2-81)

Project number Z01 HL 03725-01 PA

Project description: In a November 1981 article published in the New England Journal of Medicine, St. John Sutton and his associates described a study conducted in the Brompton Hospital, London, to determine the usefulness of routine cardiac catheterization before operative replacement of one or both left-side cardiac valves. The present paper was a rebuttal to that article providing reasons why cardiac catheterization should be performed routinely except in emergency situations in patients undergoing valve replacement.

Publications: Roberts, W.C.: Sounding Board: Reasons for cardiac catheterization before cardiac valve replacement. NEJM 306:1291, 1982.

884

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03726-01 PA

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
Complications of cardiac valve replacement: characteristic abnormalities of prostheses pertaining to any or specific site

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Complications of valve replacement as observed during a 21 year experience were summarized. The complications could be divided into the following groups. Complications common to any valve site and most or all presently available mechanical or bioprosthetic valves; complications limited to a particular valve site irrespective of type of prosthesis utilized; complications following combined mitral and aortic valve replacement.

885

PHS-6040
(Rev. 2-81)

Project description: Complications of valve replacement as observed during a 21 year experience were summarized. The complications could be divided into the following groups.

Complications common to any valve site and most or all presently available mechanical or bioprosthetic valves: 1) Prosthetic size disproportion. 2) Prosthetic thrombosis. 3) Para-anular prosthetic ring discontinuity (peribasilar leak). 4) Prosthetic degeneration or wear. 5) Suture overhang of prosthetic orifice. 6) Prosthetic endocarditis. 7) Entanglement of sutures beneath the occluder. 8) Incomplete removal of native valve or its calcific deposits preventing complete occluding of the prosthetic orifice by the occluder. 9) Dislodgment of portion of native cardiac valve during its excision. 10) Development of calcific deposits beneath site of attachment of a prosthetic valve ring. 11) Hemolysis of blood elements.

Complications limited to a particular valve site irrespective of type of prosthesis utilized: 1) Disruption of connection between LA and LV at mitral anulus with extravasation of blood into atrioventricular sulcus. 2) Excision of portion of LV free wall beneath papillary muscle during excision of papillary muscle with resulting rupture or aneurysmal formation. 3) Incision of LV free wall midway between mitral anulus and stump of LV papillary muscle during mitral valve excision. 4) Damage to left circumflex coronary artery or coronary sinus during insertion of mitral prosthesis or during suture obliteration of entrance into LA appendage. 5) Contact of cage or stent of mitral prosthesis with ventricular septum or LV free wall producing ventricular arrhythmia. 6) Diffuse or extensive LV fibrosis post-mitral replacement. 7) Obstruction of coronary ostium by aortic prosthetic ring or stent. 8) Intimal thickening in aortic root post-aortic valve replacement. 9) Dissection of aorta post-aortic valve replacement.

Complications following combined mitral and aortic valve replacement: 1) Contact of aortic valve poppet with stent or ring of mitral prosthesis causing AR. 2) Compression of anomalous LC by mitral and aortic prosthetic fixation rings.

Publications: Roberts, W.C.: Complications of cardiac valve replacement: characteristic abnormalities of prostheses pertaining to any or specific site. Am Heart J 103(1):113, 1982

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03727-01 PA

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Early Calcific Stenosis of Infected Pericardial Valvular Bioprostheses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Victor J. Ferrans	Chief, Ultrastructure Section,	PA NHLBI
Others:	Edward H. Williams	Division of Cardiothoracic Surg.	Univ. Texas
	Vincent R. Conti	Division of Cardiothoracic Surg.	Univ. Texas
	Akira Nishimura	Department of Cardiology	Univ. Texas
	L. Clark Stout	Department of Pathology	Univ. Texas

COOPERATING UNITS (if any)

Division of Cardiothoracic Surgery, Department of Cardiology and Department of Pathology, University of Texas Medical Branch, Galveston, Texas

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.15	0.15	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Bioprosthetic valvular failure developed within four months of implantation in two pericardial valvular bioprostheses that became infected. Electron microscopic examination disclosed calcific deposits within necrotic bacteria in the vegetations present on the bioprosthetic leaflets.

887

PHS-6040
(Rev. 2-81)

Project Description: The clinical course of a patient with bioprosthetic failure of aortic and mitral Ionescu-Shiley valves (which contain bovine pericardial leaflets) is presented. Failure occurred in less than four months and was due to infection that resulted in calcification and severe stenosis. On examination of tissue specimens by light and electron microscopy, the pathologic process was seen to be similar to that occurring in infected porcine prostheses. Bacteria were shown to become loci of calcification in vegetations present on the pericardial tissue leaflets.

Publication: Williams, E. H., Conti, V. R., Nishimura, A., Stout, L. C., and Ferrans, V. J.: Early calcific stenosis of aortic and mitral Ionescu-Shiley valves in a patient with bioprosthetic infection. A case report. J. Thorac. Cardiovasc. Surg. 82: 391-397, 1981.

SSS

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03728-01 PA

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Subcutaneous Implantation Model for Study of Bioprosthetic Valvular Calcification

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Victor J. Ferrans, Chief, Ultrastructure Section PA NHLBI

Others: Michael C. Fishbein, Dept. of Pathology, Cedars-Sinai Medical Center, Los Angeles, Calif.
Robert J. Levy, Department of Cardiology, Children's Hospital and Medical Center, Boston, Mass.
Lyle C. Dearden, Department of Anatomy, College of Medicine, University of California, Irvine, Calif.
Aws Nashef, American Edwards Laboratories, Santa Ana, Calif.
Arden P. Goodman, American Edwards Laboratories, Santa Ana, Calif.
Alain Carpentier, Laboratoire d'etude des Protheses Cardiaques, Hopital Broussais, Paris, France

COOPERATING UNITS (if any) Dept. of Pathology, Cedars-Sinai Medical Center, LA, Calif. Department of Cardiology, Children's Hosp. and Med. Center, Boston, Mass., Dept. of Anatomy, Univ. Calif., Irvine, Calif., American Edwards Laboratories, Santa Ana, Calif. and Hopital Broussais, Paris, France

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.21	0.21	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A model system, which consists of the implantation of pieces of/leaflets subcutaneously in rabbits, was developed to study the process of calcification of glutaraldehyde-treated bioprosthetic valves. In this model, significant calcification develops within 3-6 months of implantation. The calcific deposits are morphologically similar to those which occur in bioprosthetic valves implanted in intracardiac positions.

bioprosthetic

889

PHS-6040
(Rev. 2-81)

Project Description: To study the process of calcification in bioprostheses, 108 glutaraldehyde-treated porcine aortic valve leaflets were implanted subcutaneously in rabbits and removed 1 day to 6 months later; morphologic findings were correlated with biochemically determined levels of calcium (Ca++) and gamma-carboxyglutamic acid (Gla), a vitamin K-dependent Ca++-binding amino acid known to be present in a variety of tissues with pathological calcification. Gla and Ca++ levels began to increase about 2 months after implantation and increased progressively with time. Ca/Gla molar ratios were comparable to those in leaflets of bioprostheses explanted from patients, 22 to 64 months after implantation. Morphologically evident calcification began at the same time that Gla and Ca++ increases were detected biochemically and also increased in severity with time. Electron microscopy showed that calcification primarily involved the surface of collagen fibrils and the interfibrillar spaces. The biochemical and morphologic findings in this experimental system are similar to those described in calcified porcine bioprosthetic valve leaflets removed from patients, but occurred much more rapidly. As with pathological calcification of other tissues, progressive calcification was accompanied by increased Gla levels, suggesting that Gla plays a role in the calcification of the leaflets. This model allows rapid comparative evaluation of large numbers of valve leaflets maintained under similar host conditions.

Publication: Fishbein, M. C., Levy, R. J., Ferrans, V. J., Dearden, L. C., Nashef, A., Goodman, A. P., and Carpentier, A.: Calcification of cardiac valve bioprostheses. Biochemical, histologic, and ultrastructural observations in a subcutaneous implantation model system. J. Thorac. Cardiovasc. Surg. 83: 602-609, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03729-01 PA

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Myocardial Ultrastructure in Human Cardiac Hypertrophy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Victor J. Ferrans Chief, Ultrastructure Section PA NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.03	0.03	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A detailed description is presented of ultrastructural alterations resulting from hypertrophy in human hearts.

891

PHS-6040
(Rev. 2-81)

Project Description: A detailed analysis is presented of myocardial ultrastructural changes that occur in human cardiac hypertrophy. Three stages of hypertrophy are recognized: the stage of developing hypertrophy in which evidence of increased protein synthesis is present; the stage of stable hypertrophy, in which the myocytes are enlarged but may be qualitatively normal (although they may show quantitative abnormalities), and the stage of cellular exhaustion, in which degenerative abnormalities are present. Study of several hundred hypertrophied human hearts shows that the severity of the degenerative changes is related to the severity and duration of the heart disease.

Publications:

Ferrans, V. J.: Hipertrofia Miocardica (myocardial hypertrophy). In: Balbarrey, H. (Ed.): Primer Simposio Argentino de Miocardiopatias. Federacion Argentina de Cardiologia, Rosario, Argentina, 1981, pp. 8-13.

Ferrans, V. J.: Human cardiac hypertrophy: structural aspects. Eur. Heart J. 3: 15-27, 1982

892

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03730-01 PA

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Ultrastructural Pathology of the Heart

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Victor J. Ferrans Chief, Ultrastructure Section PA NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.03	0.03	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This is a monograph which presents a comprehensive review of our present knowledge of the ultrastructure of normal and diseased hearts, with emphasis on diagnostic applications of electron microscopy.

893

PHS-6040
(Rev. 2-81)

Project Description: This monograph is divided into the following seven sections: normal myocardial ultrastructure, myocardial hypertrophy, cardiomyopathies, myocardial ischemia, myocarditis, acute rheumatic fever and chronic rheumatic heart disease, and other diseases of the cardiac valves and endocardium. Each of these sections is extensively illustrated and presents a broad coverage, with emphasis on features that are useful for diagnostic purposes.

Publication: Ferrans, V. J.: The heart. In Johannessen, J. V. (Ed.): Electron Microscopy in Human Medicine, Volume 5. New York, McGraw-Hill, 1982, pp. 1-83.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03731-01 PA

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Myocardial Ultrastructure in the Cardiomyopathies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Victor J. Ferrans, Chief,	Ultrastructure Section	PA	NHLBI
Others:	Jagdish W. Butany	Ultrastructure Section	PA	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.06	0.06	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A detailed description is given of myocardial ultrastructural changes in the various types of cardiomyopathies, including: hypertrophic cardiomyopathy, idiopathic ventricular dilated cardiomyopathy, histiocytoid cardiomyopathy, anthracycline-induced cardiomyopathy, the syndromes of endomyocardial disease and hypereosinophilia, and various infiltrative cardiomyopathies.

895

PHS-6040
(Rev. 2-81)

Project Description: A review is presented of the ultrastructural features of the heart in the major types of cardiomyopathies. The heart in hypertrophic cardiomyopathy shows disarray of muscle cells and of myofibrils; such changes can be found, albeit to a lesser extent, in other types of heart disease. In idiopathic cardiomyopathy of the congestive or ventricular-dilated type, the heart shows hypertrophy, fibrosis and nonspecific degenerative alterations. More distinctive changes are present in two other cardiomyopathies, anthracycline-induced cardiomyopathy and infantile cardiomyopathy with histiocytoid change in the muscle cells. Endocardial and endothelial damage with mural and microvascular thrombosis (possibly related to the release of eosinophil products) appear to be the pathogenetic mechanisms in the syndromes of hypereosinophilia and endomyocardial disease. The infiltrative cardiomyopathies show a variety of changes, the nature of which depends on the type and localization (intracellular or interstitial) of the infiltrating material.

Publication: Ferrans, V. J., and Butany, J. W.: Ultraestructura del miocardio en las miocardiopatias. Revista Latina de Cardiologia 2: 456-468, 1981

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03732-01 PA

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Overview of Morphologic Reactions of the Heart to Toxic Injury

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Victor J. Ferrans, Chief, Ultrastructure Section PA NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.03	0.03	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

An extensive review was made of the morphologic abnormalities which develop in the heart and blood vessels as consequences of different types of toxic injury.

897

PHS-6040
(Rev. 2-81)

Project Description: An extensive review was made of cardiac morphologic changes caused by various toxic agents. Among these changes are: cardiac hypertrophy and dilatation; various types of cardiac cellular damage; cardiac necrosis; acute, subacute and chronic inflammation; and drug-induced vasculitis and hypersensitivity angiitis.

Publication: Ferrans, V. J.: Overview of morphologic reactions of the heart to toxic injury. In Balazs, Tibor (Ed.): Cardiac Toxicology, Volume III, Boca Raton, Florida, CRC Press, Inc., 1981, pp. 83-109.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03733-01 PA

PERIOD COVERED
October 1, 1981, to September 30, 1982.

TITLE OF PROJECT (80 characters or less)
A survey of the causes and consequences of pericardial heart disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: William C. Roberts, Chief, Pathology Branch, NHLBI

OTHER: Victor J. Ferrans, Chief, Ultrastructure Section, NHLBI

COOPERATING UNITS (if any)
Department of Medicine, Georgetown University, Washington, D.C.

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This report reviews morphologic aspects of pericardial heart disease. A morphologic classification for this condition is presented. An ideal classification of pericardial heart disease obviously would take into account clinical, etiologic, and morphologic features of this condidtion, but a single classification combining these three components is lacking. Pericardial heart disease is relatively uncommon clinically and when present at necropsy, it usually has not been recognized during life. The term "pericarditis" is inaccurate because most pericardial diseases are noninflammatory in nature. Morphologically, chronic pericardial heart disease may present clinically as an acute illness. Even when clinical symptoms are present, however, few patients develop evidence of cardiac dysfunction (constriction). When pericardial "constriction" occurs, it is the result of increased pericardial fluid or increased pericardial tissue or both. Increased fluid is treated by drainage; increased tissue, by excision. In most patients with chronic constrictive "pericarditis," the etiology is not apparent, even after histologic examination of pericardia.

899

Project description: This report reviews morphologic aspects of pericardial heart disease. A morphologic classification for this condition is presented. An ideal classification of pericardial heart disease obviously would take into account clinical, etiologic, and morphologic features of this condition, but a single classification combining these three components is lacking. Pericardial heart disease is relatively uncommon clinically and when present at necropsy, it usually has not been recognized during life. The term "pericarditis" is inaccurate because most pericardial diseases are noninflammatory in nature. Morphologically, chronic pericardial heart disease may present clinically as an acute illness. Even when clinical symptoms are present, however, few patients develop evidence of cardiac dysfunction (constriction). When pericardial "constriction" occurs, it is the result of increased pericardial fluid or increased pericardial tissue or both. Increased fluid is treated by drainage; increased tissue, by excision. In most patients with chronic constrictive "pericarditis," the etiology is not apparent, even after histologic examination of pericardia.

Publications: Roberts, W.C. and Ferrans, V.J.: A survey of the causes and consequences of pericardial heart disease. In: Pericardial Disease, edited by P.S. Reddy et al., Raven Press, New York, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03734-01 PA

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Pathology of Pericardial Effusion

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Victor J. Ferrans, Chief, Ultrastructure Section	PA	NHLBI
Others:	W. C. Roberts, Chief, Pathology Branch	PA	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.06	0.06	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A systematic review of the various types of pericardial effusion is presented, with emphasis on features that are useful in the diagnosis of pericardial diseases.

901

PHS-6040
(Rev. 2-81)

Project Description: Pericardial effusions can be classified according to the characteristics of the pericardial fluid. Identification of these characteristics is of crucial importance for the diagnosis and management of pericardial diseases. The fluid can be: 1) serous, 2) serofibrinous, 3) purulent, 4) bloody, 5) rich in cholesterol crystals (i.e., the "gold paint" fluid of cholesterol pericarditis), and 6) chylous. Distinctions between these types of fluid are made on the basis of examination of pericardial fluid for: specific gravity; protein, glucose and cholesterol content; hematocrit; total blood cell count and differential count; microscopic study for bacteria, fungi, parasites and malignant cells, and appropriate microbiological cultures and immunological studies. Additional information can be obtained by morphological and microbiological study of tissue obtained by pericardial biopsy.

Publication: Ferrans, V. J., and Roberts, W. C.: Pathology of pericardial effusion. In Reddy, P. S., Leon, D. F. and Shaver, J. A. (Eds.): Pericardial Disease. New York, Raven Press, 1982, pp. 77-92.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03735-01 PA

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Lack of correlation between clinical and morphologic findings in patients treated with anthracycline chemotherapy.....

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Jeffrey M. Isner, Deptartment of Cardiology, Tufts Univ. Medical Center

OTHER: Victor J. Ferrans, Chief, Ultrastructure Section, Pathology Branch, NHLBI
Steven R. Cohen, Surgery Branch, NHLBI
Bruce G. Witkind, Visiting Fellow, University of Miami, Miami, Florida
Renu Virmani, Department of Pathology, Vanderbilt University Med. Center
John S. Gottdiener, Senior Investigator, Cardiology Branch, NHLBI
William C. Roberts, Chief, Pathology Branch, NHLBI
J. Robert Beck, National Cancer Institute, NIH

COOPERATING UNITS (if any)
Department of Pathology, Vanderbilt University Medical Center,,Tufts University Medical Center , NCI

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The relationship between clinical evidence of anthracycline cardiotoxicity and histologic signs of anthracycline cardiotoxicity was evaluated by reviewing the clinical and morphologic findings in 64 patients studied at necropsy, all of whom had received doxorubicin or daunorubicin chemotherapy during life. Of the 64 patients, 20 (31%) had documented clinical toxicity consisting of impaired left ventricular systolic performance: in 7 (35%) of these 20 patients, histologic signs of toxicity were absent. In the remaining 13 patients, histologic signs of toxicity ranged from mild to severe. Of the 44 (69%) patients without clinical signs of drug toxicity, 21 (48%) had no histologic signs of cardiotoxicity.

PHS-6040
(Rev. 2-81)

Project description: The relationship between clinical evidence of anthracycline cardiotoxicity and histologic signs of anthracycline cardiotoxicity was evaluated by reviewing the clinical and morphologic findings in 64 patients studied at necropsy, all of whom had received doxorubicin or daunorubicin chemotherapy during life. Of the 64 patients, 20 (31%) had documented clinical toxicity consisting of impaired left ventricular systolic performance: in 7 (35%) of these 20 patients, histologic signs of toxicity were absent. In the remaining 13 patients, histologic signs of toxicity ranged from mild to severe. Of the 44 (69%) patients without clinical signs of drug toxicity, 21 (48%) had no histologic signs of cardiotoxicity; in 23 (52%) of the patients without clinical toxicity, however, morphologic signs of cardiotoxicity were nevertheless present, mild in most patients, but extensive in four. Signs of extensive histologic toxicity (19 [30%] of 64 patients) were associated with large doses (>450 mg/M2) of drug, mediastinal irradiation, and age >70 years old.

The results of this study suggest that attempts to monitor cardiotoxicity by serial evaluation of cardiac histology in patients undergoing anthracycline chemotherapy may be seriously limited by the fact that clinical evidence of toxicity may be present without histologic signs of toxicity; likewise, histologic signs of anthracycline toxicity may be present without clinical evidence of toxicity.

Publications: Isner, J.M., Ferrans, V.J., Cohen S.R., Witkind B.G., Virmani, R, Gottdiener J.S., Roberts, W.C., Beck. J.R.: Lack of correlation between clinical and morphologic findings in patients treated with anthracycline chemotherapy. An analysis of 64 patients studied at necropsy. American Journal of Medicine (submitted)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03736-01 PA

PERIOD COVERED
October 1, 1981, to September 30, 1982.

TITLE OF PROJECT (80 characters or less)
Osteogenic sarcoma metastatic to the heart.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Katherine A. Seibert, Medicine Branch, NCI

OTHERS: Carl W. Rettenmier, Pathology Branch, NCI
Bruce F. Waller, Associate Staff Fellow, Pathology Branch, NHLBI
William E. Battle, Cardiology Branch, NHLBI
Arthur S. Levine, Medicine Branch, NCI
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)
Cardiology Branch; Pediatric, Medicine and Pathology Branches, National Cancer Institiue, Bethesda Maryland.

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Of 20 necropsy patients with fatal osteogenic sarcoma, 4 had metastases to the heart. One had recurrent ventricular tachycardia, 1 had anatomic evidence of aortic regurgitation due to massive periaortic neoplastic infiltration which prevented coaptation of the aortic cusps during ventricular diastole, 1 had massive invasion of the peri-superior vena cava tissues and typical clinical features of the superior vena caval syndrome, and one had neoplastic obstruction of the inferior vena cava as it entered the thorax. Among patients with either primary or secondary neoplasms to the heart, osteogenic sarcoma is unique because the metastases contain bone and, therefore, may be radiographically visible, they are usually large and often intracavitary.

PHS-6040
(Rev. 2-81)

Project number Z01 HL 03736-01 PA

Project description: Of 20 necropsy patients with fatal osteogenic sarcoma, 4 had metastases to the heart. One had recurrent ventricular tachycardia, 1 had anatomic evidence of aortic regurgitation due to massive periaortic neoplastic infiltration which prevented coaptation of the aortic cusps during ventricular diastole, 1 had massive invasion of the peri-superior vena cava tissues and typical clinical features of the superior vena caval syndrome, and one had neoplastic obstruction of the inferior vena cava as it entered the thorax. Among patients with either primary or secondary neoplasms to the heart, osteogenic sarcoma is unique because the metastases contain bone and, therefore, may be radiographically visible, they are usually large and often intracavitary.

Publications: Siebert, K.S., Rettenmier, C.W., Waller, B.F., Battle, W.E., Levine, A.S., and Roberts, W.C.: Osteogenic sarcoma metastatic to the heart. Am. J. Med., July 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03737-01 PA

PERIOD COVERED: October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Total anomalous pulmonary venous connection: survival for 62 years without surgical intervention

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Bruce M. McManus, Senior Staff Fellow, Pathology Branch, NHLBI

OTHER: Josef Luetzeler, Department of Pathology, Holy Cross Hospital
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

Department of Pathology, Holy Cross Hospital

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Most patients with total anomalous pulmonary venous connection (TAPVC), irrespective of the drainage site, live for less than 6 months. Of reported nonoperated necropsy patients with TAPVC with drainage of a common pulmonary vein into the left innominate vein via a left vertical vein ("Snowman" type), none had survived as long as 10 years. The oldest reported patient with TAPVC of any type with necropsy verification was 39 years of age. Recently, we studied at necropsy a 62-year-old man with unoperated TAPVC with drainage of a retro-atrial common vein into the left innominate vein.

907

Project description: Most patients with total anomalous pulmonary venous connection (TAPVC), irrespective of the drainage site, live for less than 6 months. Of reported nonoperated necropsy patients with TAPVC with drainage of a common pulmonary vein into the left innominate vein via a left vertical vein ("Snowman" type), none had survived as long as 10 years. The oldest reported patient with TAPVC of any type with necropsy verification was 39 years of age. Recently, we studied at necropsy a 62-year-old man with unoperated TAPVC with drainage of a retroatrial common vein into the left innominate vein. He was cyanotic shortly after birth, acyanotic from about age 20 to 40 years, and cyanotic again during approximately his last 20 years. He was dyspneic on moderate exertion during his last 20 years. He had an acute febrile illness at age 48 years characterized by cough and excessive dyspnea. At that time chest roentgenogram disclosed cardiomegaly and a cavity in the left upper lobe. The pulmonic second sound was increased in intensity. After antibiotic therapy the signs suggestive of infection vanished. Because of cyanosis and cardiomegaly he underwent cardiac catheterization 6 months later. Pressures (mm Hg) and oxygen saturations (%) respectively were femoral artery (130/90, 78), left ventricle (110/3, 78), right ventricle (94/5, 78), left atrium (5, 76), right atrium (6, 78); oxygen saturations (%) superior vena cava (90), low left innominate vein (92), left subclavian vein (77), left brachial vein (59); and cardiac output 5.6 L/min. The ECG at that time and 2 days before death are shown in Fig. 2. He never had overt congestive cardiac failure or a precordial murmur. He died from complications of a perforated duodenal ulcer.

Besides lack of excessive pulmonary vascular resistance, survival in TAPVC appears to be dependent primarily on three factors: (1) the size of the defect in the atrial septum; (2) the length of the anomalous pulmonary veins(s), and (3) the degree of obstruction to flow in the anomalous pulmonary vein(s). Our patient appeared to have survived so long because of nearly ideal characteristics of each of these three factors. The atrial septum was virtually absent and therefore there was no interference to flow to the left side of the heart. The anomalous pulmonary vein was relatively short and free of any degree of obstruction. The cause of the total thrombotic occlusion of the left main pulmonary artery in our patient was not determined. It is clear, however, that this artery was totally occluded for at least 13 years and maybe considerably longer. Its occlusion in the presence of the left-to-right shunt (via the TAPVC) may have further elevated the pulmonary arterial pressures.

Publications: McManus, B.M., Luetzeler, J., Roberts, W.C.: Total anomalous pulmonary venous connection: survival for 62 years without surgical intervention. Am Heart J 103(2):298, 1982

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03738-01 PA

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)
The case for preoperative coronary angiography in patients with Tetralogy of Fallot and other complex congenital heart diseases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Bruce M. McManus, Senior Staff Fellow, Pathology Branch, NHLBI

OTHER: Bruce F. Waller, Associate Staff Fellow, Pathology Branch, NHLBI
Michael Jones, Surgery Branch, NHLBI
Stephen E. Epstein, Chief, Cardiology Branch, NHLBI
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)
Surgery Branch, NHLBI, Cardiology Branch, NHLBI

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

During a recent 4-month period we studied two patients who died shortly after operative "repair" of either tetralogy of Fallot (TF) or double-outlet right ventricle (RV), both with subpulmonic obstruction, and necropsy in each disclosed that a major epicardial coronary artery had been inadvertently severed at operation. Because neither patient had had coronary angiography preoperatively and because death in each appeared to have resulted from transection of a major coronary artery, a discussion of the rationale for coronary angiography before operative "correction" of complex congenital heart disease appeared warranted.

909

PHS-6040
(Rev. 2-81)

Project description: During a recent 4-month period we studied two patients who died shortly after operative "repair" of either tetralogy of Fallot (TF) or double-outlet right ventricle (RV), both with subpulmonic obstruction, and necropsy in each disclosed that a major epicardial coronary artery had been inadvertently severed at operation. Because neither patient had had coronary angiography preoperatively and because death in each appeared to have resulted from transection of a major coronary artery, a discussion of the rationale for coronary angiography before operative "correction" of complex congenital heart disease appeared warranted.

Each of the above two patients demonstrates the disastrous consequences of severing a major coronary artery during operations for complex congenital heart disease. Neither patient preoperatively had either selective coronary or aortic root angiography, and at operation the anomalous coronary artery was not identified before being severed because of either overlying epicardial adhesions or excessive adipose tissue or both. Thus despite repair of the complex anomaly (TF or double-outlet RV with subpulmonic obstruction), the presence of a simple anomaly (coronary artery) which was unidentified and inadvertently severed proved fatal in each. How can this fatal complication be prevented? One approach would be to perform coronary angiography preoperatively in all patients with complex congenital heart disease (in whom ventriculotomy is planned) and at least five reasons appear to make this approach justifiable.

1) An operatively important coronary anomaly is sufficiently frequent in patients with many complex congenital cardiac anomalies.

2) An anomalous coronary artery coursing across the RV outflow tract may not be identifiable at operation.

3) Damage to a coronary artery at operation may have serious or fatal consequences.

4) Knowledge of the presence of a significant coronary anomaly may alter the timing and type of operative procedure performed.

5) Coronary angiography is a safe procedure in these patients.

Publications: McManus, B.M., Waller, B.F., Jones, M., Epstein, S.E., and Roberts, W.C.: The case for preoperative coronary angiography in patients with tetralogy of Fallot and other complex congenital heart diseases. Am Heart J 103(3):451, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03739-01 PA

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Lesions of Selenium-Vitamin E Deficiency in Ducklings Fed Trace Elements

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Victor J. Ferrans	Chief, Ultrastructure Section PA NHLBI
Others:	John F. VanVleet	Professor, Dept. of Microbiology, Pathology and Public Health, School of Veterinary Medicine, Purdue Univ.
	G. D. Boon	Dept. of Microbiology, Pathology and Public Health School of Veterinary Medicine Purdue University

COOPERATING UNITS (if any)

School of Veterinary Medicine, Purdue University, West Lafayette, Ind.

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.09	0.09	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The interactions between different trace elements were studied in ducklings given diets that were adequate in selenium and vitamin E (Se-E) but contained increased amounts of either Ag, Cu, Co, Te, Cd or Zu. These animals developed cardiac lesions which were morphologically similar to those of Se-E deficiency and were prevented by concurrent administration of Se-E.

911

PHS-6040
(Rev. 2-81)

Project Description: In 3 experiments 684 newly-hatched White Pekin ducklings were fed for 15 to 28 days a commercial starter mash that was adequate in selenium and vitamin E (Se-E) content, either alone or with supplements of Ag (3000 ppm as acetate), Cu (1500 ppm as sulfate), Co (200 or 500 ppm as chloride), Te (500 ppm as tetrachloride), Cd (500 ppm as sulfate), Zn (3000 or 6000 ppm as sulfate) or V (100 ppm as vanadate). The ducklings fed Ag, Cu, Co, Te, Cd, and Zn frequently developed lesions characteristics of Se-E deficiency, such as necrosis of skeletal and cardiac muscle and of smooth muscle of the gizzard and intestine. Complete protection from the muscle lesions produced by Cu, Co, Te, Cd, and Zn supplements was provided by vitamin E (200 IU α-tocopherol acetate/Kg) and selenium (2 ppm as selenite). Ducklings fed Ag were protected by supplements of vitamin E and partial protection was achieved by selenium addition. The birds fed excess Zn developed pancreatic necrosis and fibrosis that was not prevented by supplements of selenium or vitamin E. Terminally, blood glutathione peroxidase (GSH-Px) activity was low and hepatic selenium content was elevated in Ag-fed ducklings. However, neither blood GSH-Px activity or hepatic selenium content were consistently abnormal in ducklings fed other trace elements, although lesions of Se-E deficiency were often present in these animals.

Publication: VanVleet, J. F., Boon, G. D., and Ferrans, V. J.: Induction of lesions of selenium-vitamin E deficiency in ducklings fed silver, cobalt, cadmium, copper, tellurium or zinc and protection by selenium or vitamin E supplements. Am. J. Vet. Res. 42: 1206-1217, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03740-01 PA

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Effects of propranolol and furosemide on minoxidil-induced cardiomyopathy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Victor J. Ferrans	Chief, Ultrastructure Section	PA NHLBI
Others:	Eugene H. Herman	Division of Drug Biology	FDA
	Tibor Balazs	Division of Drug Biology	FDA
	Robert S. K. Young	Division of Drug Biology	FDA

COOPERATING UNITS (if any)
Division of Drug Biology, Food and Drug Administration

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.12	0.12	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The acute cardiomyopathy induced in beagle dogs by the administration of minoxidil, a vasodilating antihypertensive agent, is prevented by pretreatment with furosemide but not by pretreatment with propranolol or with hydrochlorothiazide.

913

PHS-6040
(Rev. 2-81)

Project Description: Previous studies have shown that oral administration of minoxidil on 2 consecutive days produces an increase in heart rate and myocardial necrosis in Beagle dogs. Propranolol treatment (1.0 or 3.0 mg/kg every 8 h) did not abolish tachycardia and did not alter the incidence or severity of minoxidil-induced necrosis. Pretreatment with either furosemide (10 mg/kg) or hydrochlorothiazide (250 mg/kg) reduced serum potassium levels. However, only furosemide (for 11 days) reduced significantly the incidence of minoxidil-induced necrosis; only 2 of 10 animals (20%) developed myocardial lesions compared to 11 of 14 (79%) in the non-treated group. The incidence and severity of lesions in hearts from animals treated with furosemide for 3 days or hydrochlorothiazide for 11 days were essentially the same as in animals given minoxidil alone.

Publications: Herman, E. H., Balazs, T., Ferrans, V. J., and Young, R. S. K.: Divergent effects of propranolol and furosemide pretreatment on acute cardiomyopathy induced by minoxidil in beagle dogs. Toxicology 20: 155-164, 1981.

Herman, E. H., Ferrans, V. J., and Balazs, T.: Minoxidil and cardiac lesions. Circulation 64: 1299-1300, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03741-01 PA

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Mechanisms of Hydralazine-induced Myocardial Necrosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Victor J. Ferrans	Chief, Ultrastructure Section	PA	NHLBI
Others:	Tibor Balazs	Division of Drug Biology		FDA
	A. El-Hage			FDA
	S. J. Ehrreich			FDA
	G. L. Johnson			FDA
	Eugene H. Herman	Division of Drug Biology		FDA
	J. C. Atkinson			FDA
	W. L. West	Dept. of Pharmacology Howard University College of Medicine		

COOPERATING UNITS (if any)
Food and Drug Administration, Howard University College of Medicine

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.24	0.24	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Morphologic observations and data on ^{45}Ca uptake indicate that the myocardial necroses produced by large doses of hydralazine, a vasodilating anti-hypertensive agent, are: (1) related to tachycardia, hypoxia and increased flux of calcium into cardiac muscle cells, and (2) prevented by propranolol or verapamil.

915

PHS-6040
(Rev. 2-81)

Project Description: Hydralazine, 25 mg/kg ip, given on 2 consecutive days, causes myocardial necrosis in rats. Propranolol, 10 mg/kg sc twice daily, or verapamil, 10 mg/kg sc twice daily, protected against this effect. Hydralazine alone caused marked tachycardia, but bradycardia occurred when hydralazine and propranolol were given in combination. Verapamil caused a mild tachycardia which was increased further by hydralazine. Pretreatment with dihydrotachysterol, 1 mg/kg po for 3 days, aggravated the hydralazine-induced lesion. ^{45}Ca measurements indicated increased calcium concentration in the heart 6 hr after hydralazine administration. The data suggest that the lesion is induced by the pharmacological effects of hydralazine and that hypoxia and increased intracellular calcium play a role in the development of necrosis.

Publication: Balazs, T., Ferrans, V. J., El-Hage A., Ehrreich, S. J., Johnson, G. L., Herman, E. H., Atkinson, J. C. and West, W. L.: Study on the mechanism of hydralazine-induced myocardial necrosis in the rat. Toxicol. Appl. Pharmacol. 59: 524-534, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03742-01 PA

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Protective Effect of ICRF-187 Against Anthracycline-induced Cardiotoxicity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Victor J. Ferrans	Chief, Ultrastructure Section	PA NHLBI
Others:	Eugene H. Herman	Division of Drug Biology	FDA

COOPERATING UNITS (if any)

Food and Drug Administration

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.06	0.06	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The concurrent administration of small doses of ICRF 187 ((±)-1,2 bis(3,5-dioxopiperazinyl-1-yl) propane) causes a very significant reduction in the incidence and severity of the chronic cardiomyopathy caused in rabbits and dogs by antineoplastic agents of the anthracycline type (doxorubicin and daunorubicin).

917

PHS-6040
(Rev. 2-81)

Project Description: The potential protective effect of ICRF-187 against the chronic cardiomyopathy induced by daunorubicin and doxorubicin was examined in rabbits and beagle dogs. Rabbits were given 3.2 mg/kg daunorubicin alone or 30 minutes after 12.5 or 25 mg/kg ICRF-187 at 3 week intervals (5 injections). Beagle dogs were given doxorubicin (1.0 mg/kg) either alone or 30 minutes after 12.5 mg/kg ICRF-187 at weekly intervals (15 injections). Lesions consisting mainly of vacuolization and myofibrillar loss were noted in the hearts of all 12 rabbits and 6 dogs given the anthracyclines alone. In contrast, no abnormalities were found in 4 of 12 rabbits and 4 of 6 dogs pretreated with ICRF-187. The remaining rabbit and dog hearts from the pretreatment groups had minimal alterations. Serum iron concentration was decreased in all animals treated with the anthracyclines with or without ICRF-187. These studies demonstrate that the concurrent administration of ICRF-187 offers a practical means of reducing chronic daunorubicin and doxorubicin cardiac toxicity under circumstances similar to those in which these drugs are used for cancer chemotherapy in humans.

Publications:

Herman, E. H., and Ferrans, V. J.: ICRF-187 reduction of chronic daunorubicin and doxorubicin cardiotoxicity in rabbits and beagle dogs. Chemioterapia Oncologica 5 (1): 37-41, 1981.

Herman, E. H., and Ferrans, V. J.: Reduction of chronic doxorubicin cardiotoxicity in dogs by pretreatment with (±)-1,2 bis(3,5-dioxopiperazinyl-1-yl) propane (ICRF-187). Cancer Res 41: 3436-3440, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03743-01 PA

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Occurrence of Endothelial Cells in Implanted Valvular Bioprostheses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Victor J. Ferrans, Chief, Ultrastructure Section	PA	NHLBI
Others:	Tokuhiro Ishihara, Ultrastructure Section	PA	NHLBI
	Michael Jones Surgery Branch	SU	NHLBI
	Steven W. Boyce Ultrastructure Section	PA	NHLBI
	William C. Roberts, Chief, Pathology Branch	PA	NHLBI

COOPERATING UNITS (if any)

Surgery Branch, NHLBI

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.15	0.15	

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Histologic and scanning electron microscopic studies were made to determine the frequency of occurrence of endothelial cells on the surfaces of porcine valvular bioprostheses implanted in 43 patients. Endothelial cells were found to grow on the surfaces of bioprostheses as a function of time elapsed after implantation in the atrioventricular position; such cells were not found in bioprostheses that had been implanted in the aortic position. The growth of endothelial cells may serve to increase the structural stability of bioprosthetic cusps.

PHS-6040
(Rev. 2-81)

Project Description: Histologic and scanning and transmission electron microscopic studies were made to determine the frequency of occurrence of endothelial cells in 49 porcine valvular bioprostheses removed from 43 patients at times ranging from 2 days to 113 months (average, 35 months) after implantation. Endothelial cells were found in none of 17 bioprostheses in place for less than 1 year; in 5 (23%) of 22 in place for 1 to 5 years, and in 7 (70%) of 10 in place for longer than 5 years.

The 12 bioprostheses in which endothelial cells were present had been implanted in the atrioventricular position (7 of 32 in the mitral position and 5 of 6 in the tricuspid position) for periods of time ranging from 21 to 113 months (average, 71 months). Endothelial cells were not found in any of 11 bioprostheses implanted in the semilunar position (9 in the aortic position and 2 in pulmonic conduits); however, all but one of these had been in place for less than 5 years. Endothelial cells were concentrated along the basal regions of the cusps. These cells did not grow in direct contact with valvular collagen, but were attached to fibrin, thrombi or fibroelastic host tissue (fibrous sheath) on the valvular surfaces. The growth of endothelial cells and associated fibrous tissue may serve to increase the structural stability of bioprosthetic cusps, and this may be of functional importance several years after implantation, by which time the porcine connective tissue may have undergone significant deterioration.

Publication: Ishihara, T., Ferrans, V. J., Jones, M., Boyce, S. W. and Roberts, W. C.: Occurrence and significance of endothelial cells in implanted porcine bioprosthetic valves. Am. J. Cardiol. 48: 443-454, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03744-01 PA

PERIOD COVERED: October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Morphology of Cuspal Tears and Perforations in Implanted Bioprosthetic Valves

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Victor J. Ferrans	Chief, Ultrastructure Section	PA	NHLBI
Others:	Tokuhiro Ishihara	Ultrastructure Section	PA	NHLBI
	Steven W. Boyce	Ultrastructure Section	PA	NHLBI
	Michael Jones	Surgery Branch	SU	NHLBI
	William C. Roberts	Chief, Pathology Branch	PA	NHLBI

COOPERATING UNITS (if any)

Surgery Branch, NHLBI

LAB/BRANCH: Pathology Branch

SECTION: Ultrastructure Section

INSTITUTE AND LOCATION: NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.15	0.15	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Morphologic studies of cuspal tears and perforations developing in implanted cardiac valvular bioprostheses led to the conclusion that such lesions result from structural failure of connective tissue components, particularly of collagen. A simple, practical system is proposed to classify the various types of cuspal lesions observed.

921

PHS-6040
(Rev. 2-81)

Project Description: Morphologic studies were made of cuspal tears and perforations in 16 porcine valve bioprostheses that had been implanted in 14 patients (9 male and 5 female) ranging in age from 2 to 65 years. Eleven bioprostheses had been in the mitral position for 30 to 123 months, four in the aortic position for 15 to 40 months and one in a valved pulmonary conduit for 96 months. The cuspal lesions were classified into four types. Type I lesions, which involved the free edges of the cusps, were the most common of all lesions and occurred with equal frequency in mitral and aortic bioprostheses. Regardless of position of implantation, type I lesions were more frequent in the right coronary cusp than in the other cusps. Ultrastructural study showed that these lesions develop as consequences of breakdown of collagen at the free edges of the cusps, usually near the commissures. Type II lesions consisted of linear perforations that extended along the basal regions of the cusps, forming an arc parallel to the sewing ring. These lesions were uncommon and resulted from separation of bundles of collagen. Type III lesions, which were large, round or oval perforations that occupied central regions of the cusps, were more common in aortic than in mitral bioprostheses. They were characterized by marked destruction of cuspal tissue and were most frequently associated with infection. Type IV lesions were small pinhole-like perforations; they usually were multiple, localized in central regions of the cusps and associated with calcific deposits. Type IV lesions were more common in mitral than in aortic bioprostheses. Regardless of position, they were more frequent in the left and noncoronary cusps than in the right coronary cusp (which may be protected by its muscle shelf against this type of perforation). It is concluded that cuspal tears and perforations develop in implanted bioprostheses as consequences of structural failure of connective tissue components.

Publication: Ishihara, T., Ferrans, V. J., Boyce, S. W., Jones, M. and Roberts, W. C.: Structure and classification of cuspal tears and perforations in porcine bioprosthetic cardiac valves implanted in patients. Am. J. Cardiol. 48: 665-678, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03745-01 PA

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Protection by ICRF-187 Against Pancreatic Toxicity of Alloxan

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Victor J. Ferrans	Chief, Ultrastructure Section	PA	NHLBI
Others:	Antonine El-Hage	Division of Drug Biology,		FDA
	Eugene H. Herman	Division of Drug Biology,		FDA

COOPERATING UNITS (if any)
Division of Drug Biology, Food and Drug Administration, Washington, D. C.

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.09	0.09	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

ICRF 187, a bisdioxopiperazine derivative of EDTA, protects against the hyperglycemic and diabetogenic effect of alloxan in mice. The observations made are consistent with the hypothesis that formation of free radicals is responsible for the pancreatic damage and that ICRF 187 prevents the formation of these radicals.

923

PHS-6040
(Rev. 2-81)

Project Description: Blood glucose concentrations were markedly elevated in CD-1 mice 48 hr after iv administration of alloxan (75 mg/kg). Treatment with three doses of ICRF-187 (96 to 345 mg/kg) given 60 min before and 4 and 8 hr after alloxan significantly attentuated the increase in blood glucose. Pretreatment with dimethyl sulfoxide (DMSO), a known free radical scavenger, at doses of 3.5 to 7.3 g/kg also protected against the alloxan diabetogenic action. When the lowest doses of ICRF-187 (96 mg/kg) and DMSO (3.5 g/kg) were combined, alloxan exerted no hyperglycemic effect. The protective effects of ICRF-187 and DMSO were confirmed morphologically. In alloxan-treated animals, beta cell granules were absent. In contrast, the degree of granulation showed only a mild to moderate reduction in those alloxan-treated animals given ICRF-187 alone, DMSO alone, or the combination of ICRF-187 and DMSO. These results suggest that ICRF-187 may alter the mechanism of free radical generation thought to be responsible for the production of alloxan diabetes.

Publication: El-Hage, A. N., Herman, E. H., and Ferrans, V. J.: Reduction in the diabetogenic effect of alloxan in mice by treatment with the antineoplastic agent ICRF-187. Res. Commun. Chem. Pathol. Pharmacol. 33: 509-523, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03746-01 PA

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Structure of Alveolar Epithelial Cells in Patients With Fibrotic Lung Disorders

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Victor J. Ferrans, Chief,	Ultrastructure Section	PA	NHLBI
Others:	Oichi Kawanami	Ultrastructure Section	PA	NHLBI
	Ronald G. Crystal	Chief, Pulmonary Branch	PB	NHLBI

COOPERATING UNITS (if any)

Pulmonary Branch, NHLBI

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.09	0.09	

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Alveolar epithelial cells in patients with fibrotic lung disorders are known to undergo a metaplastic or cuboidal transformation. This study presents the first detailed description of the morphology of these cuboidal cells, and it also provides a hypothesis to explain the formation of such cells. It is concluded that two types of cuboidal cells can be recognized, that they are derived from bronchiolar epithelial cells, and that the latter cells become sources of epithelial renewal in severely damaged alveoli in fibrotic lungs.

925

Project Description: Ultrastructural studies were made of the types of alveolar epithelial cells in fibrotic lungs from 34 patients, including 20 with idiopathic pulmonary fibrosis, 5 with collagen-vascular diseases, 6 with sarcoidosis, 1 with lymphangioleiomyomatosis, 1 with histiocytosis X and 1 with chronic eosinophilic pneumonia. In 28 patients, proliferation of type II alveolar epithelial cells was recognized on the basis of lamellar bodies in the cytoplasm, microvilli in the luminal surface, focal microfoldings of the basal plasma membrane, close interaction with underlying mesenchymal cells, and unilayered arrangement. Two types of cuboidal epithelial cells were recognized and were considered to be derived from bronchiolar basal cells (type A cuboidal cells) and from cuboidal cells in respiratory bronchioles (type B cuboidal cells). Type A cuboidal cells frequently contained large numbers of cytoskeletal filaments, and their basal plasma membranes possessed hemidesmosomes in close association with anchoring fibrils. Type B cells lacked hemidesmosomes and anchoring fibrils. Proliferation of either or both types of cuboidal cells was found in 30 patients. In 10 patients (average degree of fibrosis = 3.5 on a scale of 0 to +4), the proliferation involved type A cells; in 10 other patients (average degree of fibrosis = 2.5), type B cells; in 9 patients (average degree of fibrosis = 3.4), both type A and type B cells; in 1 patient cuboidal cells were identified only by light microscopy. In 17 patients, proliferating cuboidal cells formed foci of epithelial pseudostratification. Type II alveolar epithelial cells did not participate in the process of multilayering. Thus, type II alveolar epithelial cells and 2 types of cuboidal epithelial cells, are sources of epithelial renewal in damaged alveoli in fibrotic lungs. Type II cells proliferate mainly in areas of less severe degrees of fibrosis; cuboidal cells become the main source of epithelial renewal in areas of very severe lung damage.

Publication: Kawanami, O., Ferrans, V. J., and Crystal, R. G.: Structure of alveolar epithelial cells in patients with fibrotic lung disorders. Lab. Invest. 46: 39-53, 1982

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03747-01 PA

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Intracuspal Hematomas in Implanted Porcine Bioprosthetic Valves

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Victor J. Ferrans, Chief,	Ultrastructure Section	PA	NHLBI
Others:	T. Ishihara	Ultrastructure Section	PA	NHLBI
	Glenn Barnhart	Surgery Branch	SU	NHLBI
	Michael Jones	Surgery Branch	SU	NHLBI
	Charles L. McIntosh	Surgery Branch	SU	NHLBI
	W. C. Roberts Chief,	Pathology Branch	PA	NHLBI

COOPERATING UNITS (if any)

Surgery Branch, NHLBI

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.18	0.18	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Intracuspal hematomas, characterized by accumulations of blood between the layers of the cusps of porcine aortic valvular bioprostheses, are described for the first time. These lesions represent a relatively common complication after implantation of bioprostheses, and their clinical significance varies according to the size of the hematomas and the extent to which they interfere with the mobility of the affected cusps.

927

PHS-6040
(Rev. 2-81)

Project Description: Intracuspal hematomas were found in three of 57 porcine valvular bioprostheses implanted as substitute cardiac valves in 50 patients and in 11 of 29 similar bioprostheses implanted in sheep. The three valves implanted in patients had been in the mitral position for 27, 65 and 107 months. Of the valves implanted in sheep, six had been in the mitral position and five in the tricuspid position for periods of time ranging from 20 minutes to seven months. In each patient and in four of 11 sheep the hematomas involved more than one cusp. These lesions were localized in the spongiosa, extended from the basal region towards the free edge of the cusp, and formed a plane of dissection which involved the spaces left in the spongiosa by the removal of proteoglycan material during preimplantation commercial processing. In one patient, the hematomas limited the mobility of the cusps and appeared to have been the cause of clinically significant prosthetic mitral stenosis; in the other two patients and in the experimental animals the hematomas were smaller and of uncertain hemodynamic significance. Intracuspal hematomas may become sites of eventual formation of calcific deposits. The pathogenesis of intracuspal hematomas is related to the entry of blood into the space between the sewing ring and the most basilar region of the bioprosthetic tissue. This space extends throughout the circumference of the bioprosthesis and is continuous with the spongiosa, the layer in which the hematoma develops. The blood penetrates into this space through the suture line between bioprosthetic tissue and sewing ring on the inflow surface of porcine valvular bioprostheses which have been mounted on atrioventricular type stents. Intracuspal hematomas were not found in bioprostheses mounted on aortic type stents, in which these sutures are more protected and more closely spaced, or in pericardial bioprostheses, which do not have a spongiosa.

Publication: Ishihara, T., Ferrans, V. J., Barnhart, G. R., Jones, M., McIntosh, C., and Roberts, W. C.: Intracuspal hematomas in implanted porcine valvular bioprostheses, clinical and experimental studies. J. Thorac. Cardiovasc. Surg. 83: 399-407, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03748-01 PA

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Anatomy of the Pericardium

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Victor J. Ferrans,	Chief, Ultrastructure Section	PA	NHLBI
Others:	Tokuhiro Ishihara	Ultrastructure Section	PA	NHLBI
	W. C. Roberts	Chief, Pathology Branch	PA	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.09	0.09	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The anatomy of the human pericardium, including gross anatomic, histologic and ultrastructural features is described in detail.

429

PHS-6040
(Rev. 2-81)

Project Description: This project consisted of a detailed review of the gross anatomic, histologic and ultrastructural features of parietal and visceral pericardium. Emphasis is placed on the description of new observations, made in our laboratory, on the scanning electron microscopic appearance of the pericardial serosal surfaces and on the morphologic changes that these surfaces undergo during systole and diastole.

Publication: Ferrans, V. J., Ishihara, T., and Roberts. W. C.: Anatomy of the pericardium. In Reddy, P. S., Leon, D. F. and Shaver, J. A. (Eds.): Pericardial Disease. New York, Raven Press, 1982, pp. 15-29.

Annual Report of the Pulmonary Branch
National Heart, Lung and Blood Institute
October 1, 1981 through September 30, 1982

A large proportion of chronic human lung diseases are diseases of the alveoli, hollow ball-like structures that form the parenchyma of the respiratory tract, the site of gas exchange. In general, the non-infectious, non-malignant disorders of the alveoli are grouped as (1) the interstitial lung disorders, diseases characterized by thickening of alveolar walls, and (2) the destructive lung disorders, diseases characterized by disruption and loss of alveolar walls. The research carried out by the Pulmonary Branch over the past year has focused on both the interstitial lung disorders and the destructive lung disorders.

I. The Interstitial Lung Disorders. The interstitial lung disorders are a group of heterogenous, chronic diseases of the alveolar structures characterized by marked accumulation of inflammatory and immune effector cells, changes in alveolar epithelial, endothelial, and mesenchymal cells and alterations in the alveolar connective tissue matrix usually referred to as "fibrosis."

The interstitial lung diseases are most conveniently categorized as those of known etiology and unknown etiology. The disorders of known etiology, comprising approximately 35% of all interstitial disorders, include the inhalational disorders (e.g., inorganic dusts, organic dusts, gases, fumes, vapors, and aerosols) as well as the disorders resulting from radiation, drugs, and poisons. There are many interstitial lung disorders of unknown etiology but the most commonly encountered include sarcoidosis, idiopathic pulmonary fibrosis, the chronic interstitial lung disorders associated with the collagen-vascular disorders, and histiocytosis-X.

The pathogenesis of the derangements of the alveolar structures of almost all of the interstitial lung diseases of both known and unknown etiology results from the fact that these disorders are chronic inflammatory diseases. This chronic inflammation, usually referred to as the "alveolitis" of the interstitial lung diseases, modulates the injury to the alveolar structures by virtue of the release of potent mediators from the effector cells that comprise the inflammation. In this context, current concepts of the pathogenesis of these disorders suggest that it is these mediators that modulate most of the changes to the parenchymal cells and connective tissue matrix that define the physiologic abnormalities that are characteristic of these disorders.

The alveolar macrophage, a cell derived from the mononuclear phagocyte system, comprises approximately 90% of the effector cell population of the normal alveolar structures. Whereas the normal nonsmoking human has a small number of alveolar macrophages per alveolus, this number is increased several fold in the interstitial lung diseases. Recent studies have focused on cytotoxic effector functions of alveolar macrophages in some of the interstitial lung diseases. In idiopathic pulmonary fibrosis, a disorder characterized by progressive epithelial cell damage, alveolar macrophages are capable of spontaneously injuring rat lung parenchymal cells as evidenced by the ability of these cells to induce release of ^{51}Cr from ^{51}Cr-labeled rat lung parenchymal cells. At least some of this injury is inhibited by antioxidants suggesting reactive oxidant species may be responsible for such damage. Alveolar macrophages from a variety of human interstitial lung disorders are spontaneously releasing fibronectin, a 440,000 dalton adhesive glycoprotein. While normal human

alveolar macrophages produce 0.5-2.0 ng fibronectin/10^6 cells-hr, macrophages from patients with idiopathic pulmonary fibrosis, sarcoidosis, and a variety of other disorders spontaneously releasing 5-10 fold more fibronectin in the same period of time. Consistent with this observation, bronchoalveolar lavage fluid of patients with fibrotic lung disease showed markedly increased levels of fibronectin, at least part of which is likely derived from the alveolar macrophage population. Studies of normal alveolar macrophages as well as those from patients with fibrotic lung diseases has demonstrated that alveolar macrophages are capable of producing a variety of the components of the complement system including C3, C5, and properdin factor B. However, comparison of macrophages from patients with interstitial lung diseases to those from normal individuals show that the levels of C3 production in the two groups are similar. In sarcoidosis, a disease characterized by large numbers of activated T-lymphocytes, the alveolar macrophages are spontaneously releasing interleukin-1 a mediator known to activate certain T-lymphocytes subpopulations. This observation is consistent with the concept that the alveolar macrophage plays a central role in the expansion of T-lymphocyte numbers in sarcoidosis.

Lymphocytes comprise 10% or less of the effector cell population in the normal human alveolar structures. Sixty-five to 80% of these cells are T-lymphocytes, similar to the proportion of T-lymphocytes in blood. In sarcoidosis, a granulomatous lung disorder, the proportions of T-lymphocytes in the alveolar structures is markedly enhanced. Detailed evaluation of lung T-lymphocyte populations in sarcoidosis have demonstrated that they are activated and producing a variety of mediators that modulate both granuloma formation and hyperglobuinemia. The evaluation of T-lymphocytes isolated from the lungs of patients with active sarcoidosis demonstrate they are releasing interleukin-2, a known growth factor for T-lymphocytes. Studies of the process of "antigen presentation" a mechanism by which mononuclear phagocytes present the immune system with antigen, have demonstrated that alveolar macrophages and T-lymphocytes function in a fashion similar to that of mononuclear phagocytes and T-lymphocytes from other sites. Interestingly, however, studies of this process in sarcoidosis have demonstrated it to be markedly enhanced, i.e., T-lymphocyte proliferation in response to antigen presented by autologous alveolar macrophages is 2-3 fold enhanced in sarcoidosis compared to that of normals or idiopathic pulmonary fibrosis.

Studies of patients who develop interstitial lung disease in association with beryllium exposure have demonstrated that, as with sarcoidosis, there is marked expansion of T-lymphocytes in the alveolar structures. Like sarcoidosis, these T-lymphocytes are characterized by markedly increased numbers of T-helper cells. Mononuclear cells in the lungs of patients with berylliosis proliferate in response to beryllium, suggesting that this disease is a form of hypersensitivity lung disease.

One of the classic forms of hypersensitivity pneumonitis is "pigeon breeders disease," a disorder found in individuals who frequently inhale antigens derived from pigeons. A subgroup of these individuals develop symptoms of shortness of breath, wheezing, fevers and myalgias 4 to 6 hours after exposure to pigeon antigens, but are perfectly well in a few days. Evaluation of such individuals weeks to months after such an episode reveal that, strikingly, they have a marked expansion of T-lymphocytes in the alveolar structures. In contrast to sarcoidosis and berylliosis, however, the T-lymphocytes in the lungs of such individuals are predominantly T-suppressor cells (defined by surface antigens

using monoclonal antibodies). Interestingly, their lung mononuclear cells respond to pigeon antigens and the lung B-lymphocytes are spontaneously producing a variety of immunoglobulins. These observations suggest that if these suppressor cells are functionally active, they are probably very limited to specific aspects of the immune response to these antigens.

In the normal alveolar structures, less than 1% of the effector cell populations are neutrophils. In a variety of lung disorders including idiopathic pulmonary fibrosis, familial pulmonary fibrosis, and asbestosis, the numbers of neutrophils in the lower respiratory tract are markedly increased. The primary mechanism for this neutrophil attraction to the alveolar structures is through a mediator produced by the alveolar macrophages termed "neutrophil chemotactic factor." In addition to attracting neutrophils to the alveolar structures, this chemotactic factor activates the neutrophils so that they release a variety of mediators including proteolytic enzymes and myeloperoxidase. Evaluation of the chemotactic peptide, C5a produced by the complement system as a possible alternative mechanism for neutrophil accumulation in some of the interstitial lung disorders has revealed that it likely is not involved. There are several reasons for this including: (1) complement levels and complement activity in the lungs of these patients is normal; and (2) the lower respiratory tract is relatively C5 "deficient" i.e., there is very little C5 present so that even if the classical alternative complement pathway is activated, C5a cannot be produced because there is insufficient C5 substrate for C3b to act upon. However, evaluation of the alveolar macrophages from patients with idiopathic pulmonary fibrosis demonstrate they are producing properdin factor B and that increased levels of properdin Bb are present in the epithelial fluid. Furthermore factor Bb can act back on the normal alveolar macrophage to activate it to release neutrophil chemotatic factor. Thus, the complement system may play a role in these disorders activating cellular processes that in turn produce mediators that attract neutrophil.

An animal model of asbestosis with guinea pigs has demonstrated that, following asbestos administration to the lungs, the alveolar macrophages of these animals are spontaneously releasing the neutrophil chemotactic factor and there is marked accumulation of polymorphonuclear leukocytes in the lower respiratory tract. Furthermore, normal macrophages exposed to asbestos in vitro also release this chemotactic factor. These findings are consistent with the observation that patients with asbestosis have an accumulation of neutrophils in the lung.

Like the neutrophil, less than 1% of the effector cell populations of the human alveolar structures are eosinophils. However, in a variety of interstitial disorders, including chronic eosinophilic pneumonia and a proportion of patients with idiopathic pulmonary fibrosis, sarcoidosis and histiocytosis-X, the alveolitis is characterized by 5% or more eosinophils. Recent studies have demonstrated that eosinophils have two effector processes, cytotoxicity and collagenase production, that likely establish an important role for the eosinophils in mediating lung injury in such disorders. Evaluation of guinea pig eosinophils and human eosinophils have demonstrated that these cells are capable of injuring rat lung explants, cat lung epithelial cells, human lung fibroblasts and rat pleural mesothelial cells. At least a proportion of this cytotoxicity is inhibited by antioxidants suggesting that the cellular injury is mediated by reactive oxidant species. Eosinophils from the same sources are also carrying a collagenase that is capable of cleaving type I and type III collagen. This enzyme is predominantly in an inactive form but can be activated

by non-specific proteolytic action. In addition, the cleavage products of this enzyme together with its inhibitory profile, suggests it is a classic "collagenase." One of the problems of studying the eosinophil associated disorders of the lung parenchyma is that there has been no animal model of eosinophil accumulation in the lung. Recent studies have demonstrated that administration of polymixin-B to guinea pig lung induces an increase in the number of eosinophils in the lung and that these eosinophils are cytotoxic to to lung parenchymal cells in vitro. This is consistent with morphologic observations of these animals that demonstrates interstitial disease.

One of the major characteristics of the interstitial lung disorders is that the injury is "repaired" by mesenchymal cells, primarily fibroblasts. Whereas fibroblasts comprise approximately one third of the parenchymal cell population in normal human lung, this fraction appears to be markedly increased in the interstitial lung diseases. Evaluation of the fibronectin produced by alveolar macrophages of patients with these disorders shows that the fibronectin is capable of attracting the fibroblasts and that this fibronectin is 1000-fold more potent as a chemoattractant than is plasma fibronectin. Although collagen is considered the major site of attachment of cells mediated by fibronectin, recent studies have demonstrated the Clq component of complement (which has a collagenous region) is also capable of binding fibronectin and can mediate the attachment of fibroblasts to immune complexes. Thus, in interstitial lung disorders such as idiopathic pulmonary fibrosis which is characterized by immune complexes present within the alveolar structures, these complexes may form the nidus for attachment of fibroblasts and hence the beginning of a fibrotic site.

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abundant in the fibrotic state. Recent work in the Pulmonary Branch includes the isolation of a portion of the human α2(I) gene from a human genomic library and has characterized this gene. Using "exons" from this gene as probes, evaluation of human DNA has demonstrated that there is only one α2(I) gene. Thus, if cells modulate collagen production in interstitial lung diseases by modulating type I mRNA levels the cells must do so by mechanisms by others than choosing the number of genes to be transcribed. Consistent with this observation, additional studies have used human α2(I) probes to demonstrate that the human α2(I) gene is localized only to chromosome 7.

Recent studies also have demonstrated that human fibroblasts are capable of producing a variety of prostaglandins. This is of interest because prostaglandins are known to be capable of suppressing collagen production by fibroblasts. Interestingly, bradykinin is also capable of suppressing collagen production by fibroblasts. Although the mechanism of its action is not entirely clear, at least part appears to be through the ability of bradykinin to induce fibroblasts to produce prostaglandins of the E series which "feed back" to suppress collagen production. Recent studies have also demonstrated that human lung fibroblasts are heterogeneous in both the amounts of collagen they produce and their responses to growth factors. Thus, this observation leads to the concept that the expansion of high producing fibroblast clones rather than low producing clones may explain, in part, why some individuals are more "susceptible" to fibrosis than others, i.e., some individuals have a predominance of fibroblasts that are high producers of collagen and hence are more likely to repair lung injury with an excess of scar tissue.

Although the alveolitis of the interstitial lung disease is the major mechanism modulating injury and repair, certain interstitial lung diseases are known to be initiated by direct injury to the parenchymal cells. Included in such a category is the disease caused by the inhalation of high concentrations of oxygen for extended periods and the disease caused by paraquat, a commonly used herbicide. It is known that with inhalation of 100% oxygen for 24 hours there is a loss in lung function which is reversible if the oxygen administration is stopped. In order to understand the earliest biologic changes in the lung that precede the more permanent fibrotic form of hyperoxic lung injury, studies have been carried out with normal volunteers in which oxygen has been administered for 18 hours. These studies have demonstrated that there is a 2-3 fold increase in albumin in alveolar epithelial fluid suggesting that hyperoxia for 18 hours is associated with alveolar capillary damage and subsequent "leak." In addition, alveolar macrophages recovered from such individuals demonstrate they spontaneously release both fibronectin and the alveolar macrophage derived growth factor. Although these changes are reversible if the oxygen is stopped, they suggest possible mechanisms by which these individuals develop subsequent fibrosis. It has been known for some time that ingestion of paraquat either accidently or by suicidal intent leads to progressive pulmonary fibrosis and often death within several days to weeks. Recent observations, however, have suggested that paraquat can also be absorbed through the skin and this may be an alternative route for paraquat exposure. Interestingly, although paraquat is known to be cytotoxic to lung parenchymal cells when directly applied to animal or human parenchymal cells in vitro studies with animals have demonstrated that, following paraquat administration, there is also an alveolitis that rapidly develops characterized by an increased number of macrophages and neutrophils in the alveolar structures. Furthermore, the macrophages in paraquat exposed animals are spontaneously releasing fibronectin and there is increased amounts

of fibronectin in the alveolar epithelial fluid. In addition, the macrophages are releasing the growth factor for fibroblasts suggesting this may be one mechanism by which rapid fibrosis occurs following entrance of paraquat to the body.

Morphologic evaluation of epithelial cells from lung biopsies from patients with a variety of interstitial lung diseases has shown that there are striking changes to the epithelial cell population. There is a loss of alveolar type I cells and replacement with alveolar type II cells together with bronchial epithelial cells that "grow down" into the alveoli to replace the injured type I cells. Furthermore, there is a piling up of epithelial cells to give the appearance of metaplasia.

Evaluation of patients with histiocytosis-X, an interstitial lung disease of young adults associated with wide spread cystic disease has shown that there are markedly increased numbers of Langerhans cells comprising the alveolitis. While such cells, known derivatives of the mononuclear phagocyte system, are present in the alveolar structures in other interstitial lung diseases, histiocytosis-X is the only disease in which these cells are found in the bronchoalveolar lavage. In the past, these cells could only be identified by electron microscopy but recent studies have shown that they have a cell surface antigen identified by OKT6 monoclonal antibodies. This may prove a useful indicator of disease activity in such patients. This is an important concept because, to date, no other roentenographic, physiologic, scintigraphic or lavage parameters have been found that can accurately predict which patients will deteriorate and which will not.

II. The Destructive Lung Disease. The destructive lung disorders are characterized by loss of the alveolar structures. The current concepts of the pathogenesis of these disorders are defined by the so called "protease-antiprotease" theory of emphysema. This theory holds that in normal lung, proteases (e.g., elastase) released by inflammatory and immune effector cells within the alveolar structures are balanced by antiproteases (e.g., αl-antitrypsin, a circulating antiprotease that diffuses into the lung). In the destructive lung diseases there is increasing evidence that there is an imbalance such that the proteases overpower the anti- protease systems, thus causing connective tissue destruction and loss of the alveolar structures.

The interest of the Pulmonary Branch in the destructive lung diseases has been two fold: (1) evaluating the protease-antiprotease balance in the lower respiratory tract of humans; and (2) since elastin is a primary connective tissue target in destructive lung disease, studying elastin biosynthesis and the role of elastin fragments in the pathogenesis of these disorders. The major clinical group of patients studied in relation to destructive lung diseases is the hereditary disease αl-antitrypsin deficiency. In individuals who are homozygous for this disease, emphysema develops in the fifth decade and usually is fatal.

Previous studies from our laboratory have demonstrated that patients with PiZ, αl-antitrypsin deficiency are entirely devoid of protection against neutrophil elastase in the lower respiratory tract. In addition, studies have demonstrated that the infusion of partially purified αl-antitrypsin intravenously on a once a week basis results in the reestablishment of the antielastase protection in the alveolar structures of these individuals. Studies in the past year have

demonstrated that patients with α1-antitrypsin deficiency can be divided into two groups. The first group includes individuals with clear manifestations of destructive lung disease while the second group includes those who appear to have relatively normal lungs. Evaluation of the determinants of disease expression in the PiZ individuals show that although the two groups have similar α1-antitrypsin levels in blood and lung and have similar amounts of elastase per neutropil, the group of individuals who have destructive lung disease have evidence of neutrophils in the lung, active neutrophil elastase in their alveolar epithelial fluid, and alveolar macrophages which are spontaneously releasing neutrophil chemotactic factor. In addition to neutrophil elastase, these individuals also have evidence of neutrophil myeloperoxidase in their alveolar epithelial fluid. Since such patients also have evidence of loss of alveolar parenchymal cells, the presence of myeloperoxidase may be one mechanism (i.e., oxidant mediated) by which such cells are destroyed.

Since α1-antitrypsin deficiency is a single point mutation disease involving the structure of the α1-antitrypsin molecule, it is unclear why the alveolar macrophages of these individuals are spontaneously releasing neutrophil chemotactic factor. However, in vitro studies of normal alveolar macrophages together with neutrophil elastase, neutrophil collagenase, α1-antitrypsin, α2-macroglobulin, α2-macroglobulin-elastase complex, α2-macroglobulin-collagenase complex, and α1-antitrypsin-elastase complex have demonstrated that the presence of proteases in the alveolar structures may be related to the stimulation of release of neutrophil chemotactic factor from the alveolar macrophages. In this context, neutrophil elastase stimulates human alveolar macrophages to release the neutrophil chemotatic factor. However, collagenase does not stimulate chemotactic factor release nor does α1-antitrypsin alone nor α2-macroglobulin alone. Interestingly, neutrophil elastase complexed with α1-antitrypsin will not stimulate the macrophages to release neutrophil chemotactic factor suggesting that if there are sufficient amounts of α1-antitrypsin in the alveolar structures (i.e., in the PiZ individual resulting from α1-antitrypsin replacement therapy) the macrophages would not be stimulated to release the neutrophil chemotatic factor and hence would not be attracting neutrophils to the lung. Interestingly, in vitro studies have demonstrated that elastin fragments are chemotactic for blood monocytes providing a mechanism by which the alveolitis of a disease like α1-antitrypsin deficiency (or the emphysema associated with cigarette smoking) may be maintained.

Studies with partially purified human α1-antitrypsin have demonstrated that the anti-elastase protection of the alveolar structures of PiZ individuals can be reestablished with once a week infusions of 4 grams of α1-antitrypsin. Recent evaluation of the alveolar epithelial fluid of these individuals has shown that: before the infusion active neutrophil elastase present in their alveolar structures; two days after the second week of infusion and two days after the fourth week of infusion neutrophil elastase is no longer detectable. Thus, intraveneous α1-antitrypsin not only reestablishes the balance of antiproteases in the lower respiratory tract but also inhibits the active proteases from further destruction of the alveolar structures.

Studies have continued on the use of Danazol, an impeded androgen, which has been previously shown to increase the serum α1-antitrypsin levels in PiZ homozygous individuals to approximately 50 percent above baseline levels. Several patients have now been maintained on chronic Danazol therapy and have

maintained their elevated αl-antitrypsin serum levels with very little in the way of serious side effects.

Physiologic comparisons of PiZ αl-antitrypsin deficient individuals with patients of idiopathic pulmonary fibrosis demonstrate that they have remarkable similarities in exercise-induced abnormalities even though they have very disparate diseases. The common denominator appears to be the loss of the alveolar structures. Both sets of patients significantly decrease arterial PaO_2, with exercise while maintaining constant $PaCo_2$ levels and both groups have similar maximal oxygen consumptions (both very low compared to normal individuals of the same age).

Although the neutrophil is clearly the major source of elastase in the alveolar structures of individuals who have an accumulation of that effector cell in the lower respiratory tract (i.e., αl-antitrypsin deficiency, idiopathic pulmonary fibrosis) there has been interest in the alveolar macrophage as a possible source of proteolytic enzymes in the lung. However, although animal alveolar macrophgaes release significant amounts of collagenase and elastase, human alveolar macrophages release very little and do so only after several days in culture. Furthermore, the release of this enzyme appears to be "constitutive", i.e., it cannot be stimulated by phagocytosis, immune complexes or complement components. Thus, the production of elastase by the alveolar macrophage probably plays little role in the emphysematous process except in the cases when there are markedly increased numbers of macrophages. It is important to recognize, however, that the macrophage elastase is a very different enzyme than the neutrophil enzyme. While the latter is a serine-protease, the former is a metalo-enzyme, a very unusual form of elastase.

The tight-skin mouse represents a hereditary disorder of emphysema. Physiologic studies of these animals have demonstrated that they have classic findings of emphysema and morphologic studies of their alveolar structures demonstrate clear evidence of destruction. The mechanisms of destruction are unkonwn but recent studies evaluating inflammatory and immune effector cells in the lower respiratory tract of these animals have shown that they have neutrophils whereas normals animals do not.

Major strides have been made in the development of technology to evaluate elastin gene biosynthesis. A fragment of an elastin gene has been isolated from the sheep genome. This gene has a structure similar to other genes (i.e., introns and exons) and exons are being isolated for use as probes to determine elastin gene number and gene localization. In addition, complementary DNA has been synthesized from isolated elastin mRNA and used to quantitate elastin mRNA levels in developing lung. Consistent with morphologic studies, biochemical studies indicate that the elastin levels in the developing sheep fetal lung continue to increase. This is paralleled by increased rates of biosynthesis by the tissue and by increases in the elastin mRNA levels, suggesting elastin control at the transcriptional level.

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Interstitial Lung Disease

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SUMMARY OF WORK (200 words or less - underline keywords)

Interstitial lung disorders represent 15 to 20 percent of all diseases of the alveolar structures; in most cases these disorders cause significant disability and many are fatal. Studies of the natural history, etiology, pathogenesis and therapy of these disorders have made major inroads in the understanding of these diseases. Most importantly, is the development of the concept of the inflammatory and immune effector cells are critical determinants in the pathogenic process. Evaluation of the alveolitis of these patients has led to significant insights into the processes by which the alveolar structures are injured and repaired. As part of these studies insights have been gained into the role of the alveolar macrophage, T-lymphocyte, neutrophil, and eosinophils as well as how fibroblasts are recruited, anchored and stimulated to replicate.

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The Interstitial Lung Disorders. The interstitial lung disorders are a group of heterogenous, chronic diseases of the alveolar structures characterized by marked accumulation of inflammatory and immune effector cells, changes in alveolar epithelial, endothelial, and mesenchymal cells and alterations in the alveolar connective tissue matrix usually referred to as "fibrosis."

The interstitial lung diseases are most conveniently categorized as those of known etiology and unknown etiology. The disorders of known etiology, comprising approximately 35% of all interstitial disorders, include the inhalational disorders (e.g., inorganic dusts, organic dusts, gases, fumes, vapors, and aerosols) as well as the disorders resulting from radiation, drugs, and poisons. There are many interstitial lung disorders of unknown etiology but the most commonly encountered include sarcoidosis, idiopathic pulmonary fibrosis, the chronic interstitial lung disorders associated with the collagen-vascular disorders, and histiocytosis-X.

The pathogenesis of the derangements of the alveolar structures of almost all of the interstitial lung diseases of both known and unknown etiology results from the fact that these disorders are chronic inflammatory diseases. This chronic inflammation, usually referred to as the "alveolitis" of the interstitial lung diseases, modulates the injury to the alveolar structures by virtue of the release of potent mediators from the effector cells that comprise the inflammation. In this context, current concepts of the pathogenesis of these disorders suggest that it is these mediators that modulate most of the changes to the parenchymal cells and connective tissue matrix that define the physiologic abnormalities that are characteristic of these disorders.

The alveolar macrophage, a cell derived from the mononuclear phagocyte system, comprises approximately 90% of the effector cell population of the normal alveolar structures. Whereas the normal nonsmoking human has a small number of alveolar macrophages per alveolus, this number is increased several fold in the interstitial lung diseases. Recent studies have focused on cytotoxic effector functions of alveolar macrophages in some of the interstitial lung diseases. In idiopathic pulmonary fibrosis, a disorder characterized by progressive epithelial cell damage, alveolar macrophages are capable of spontaneously injuring rat lung parenchymal cells as evidenced by the ability of these cells to induce release of ^{51}Cr from ^{51}Cr-labeled rat lung parenchymal cells. At least some of this injury is inhibited by antioxidants suggesting reactive oxidant species may be responsible for such damage. Alveolar macrophages from a variety of human interstitial lung disorders are spontaneously releasing fibronectin, a 440,000 dalton adhesive glycoprotein. While normal human alveolar macrophages produce 0.5-2.0 ng fibronectin/10^6 cells-hr, macrophages from patients with idiopathic pulmonary fibrosis, sarcoidosis, and a variety of other disorders spontaneously releasing 5-10 fold more fibronectin in the same period of time. Consistent with this observation, bronchoalveolar lavage fluid of patients with fibrotic lung disease showed markedly increased levels of fibronectin, at least part of which is likely derived from the alveolar macrophage population. Studies of normal alveolar macrophages as well as those from patients with fibrotic lung diseases has demonstrated that alveolar macrophages are capable of producing a variety of the components of the complement system including C3, C5, and properdin factor B. However, comparison of macrophages from patients with interstitial lung diseases to those from normal individuals show that the levels of C3 production in the two groups are similar. In sarcoidosis, a disease characterized by large numbers of activated T-

lymphocytes, the alveolar macrophages are spontaneously releasing interleukin-1 a mediator known to activate certain T-lymphocytes subpopulations. This observation is consistent with the concept that the alveolar macrophage plays a central role in the expansion of T-lymphocyte numbers in sarcoidosis.

Lymphocytes comprise 10% or less of the effector cell population in the normal human alveolar structures. Sixty-five to 80% of these cells are T-lymphocytes, similar to the proportion of T-lymphocytes in blood. In sarcoidosis, a granulomatous lung disorder, the proportions of T-lymphocytes in the alveolar structures is markedly enhanced. Detailed evaluation of lung T-lymphocyte populations in sarcoidosis have demonstrated that they are activated and producing a variety of mediators that modulate both granuloma formation and hyperglobuinemia. The evaluation of T-lymphocytes isolated from the lungs of patients with active sarcoidosis demonstrate they are releasing interleukin-2, a known growth factor for T-lymphocytes. Studies of the process of "antigen presentation" a mechanism by which mononuclear phagocytes present the immune system with antigen, have demonstrated that alveolar macrophages and T-lymphocytes function in a fashion similar to that of mononuclear phagocytes and T-lymphocytes from other sites. Interestingly, however, studies of this process in sarcoidosis have demonstrated it to be markedly enhanced, i.e., T-lymphocyte proliferation in response to antigen presented by autologous alveolar macrophages is 2-3 fold enhanced in sarcoidosis compared to that of normals or idiopathic pulmonary fibrosis.

Studies of patients who develop interstitial lung disease in association with beryllium exposure have demonstrated that, as with sarcoidosis, there is marked expansion of T-lymphocytes in the alveolar structures. Like sarcoidosis, these T-lymphocytes are characterized by markedly increased numbers of T-helper cells. Mononuclear cells in the lungs of patients with berylliosis proliferate in response to beryllium, suggesting that this disease is a form of hypersensitivity lung disease.

One of the classic forms of hypersensitivity pneumonitis is "pigeon breeders disease," a disorder found in individuals who frequently inhale antigens derived from pigeons. A subgroup of these individuals develop symptoms of shortness of breath, wheezing, fevers and myalgias 4 to 6 hours after exposure to pigeon antigens, but are perfectly well in a few days. Evaluation of such individuals weeks to months after such an episode reveal that, strikingly, they have a marked expansion of T-lymphocytes in the alveolar structures. In contrast to sarcoidosis and berylliosis, however, the T-lymphocytes in the lungs of such individuals are predominantly T-suppressor cells (defined by surface antigens using monoclonal antibodies). Interestingly, their lung mononuclear cells respond to pigeon antigens and the lung B-lymphocytes are spontaneously producing a variety of immunoglobulins. These observations suggest that if these suppressor cells are functionally active, they are probably very limited to specific aspects of the immune response to these antigens.

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Human lung fibroblasts produce approximately 500,000 collagen chains/hour, 90% of which are type I collagen and 10% type III. The rate of collagen production may be modulated by exogenous stimuli. One mechanism to explain this modulation is that there are a number of genes for each collagen chain, i.e., the cell has an array of identical genes from which it can choose the number it needs as templates for mRNA production at any one time. In order to evaluate this concept, it is necessary to isolate human collagen genes to use them as probes to quantitate gene number. One of the collagen chains, the $\alpha 2(I)$ chain, is one of the two component chains of type I collagen, the collagen type that is most abundant in the fibrotic state. Recent work in the Pulmonary Branch includes the isolation of a portion of the human $\alpha 2(I)$ gene from a human genomic library and has characterized this gene. Using "exons" from this gene as probes, evaluation of human DNA has demonstrated that there is only one $\alpha 2(I)$ gene. Thus, if cells modulate collagen production in interstitial lung diseases by modulating type I mRNA levels the cells must do so by mechanisms by other than choosing the number of genes to be transcribed. Consistent with this observation, additional studies have used human $\alpha 2(I)$ probes to demonstrate that the human $\alpha 2(I)$ gene is localized only to chromosome 7.

Recent studies also have demonstrated that human fibroblasts are capable of producing a variety of prostaglandins. This is of interest because prostaglandins are known to be capable of suppressing collagen production by fibro-

blasts. Interestingly, bradykinin is also capable of suppressing collagen production by fibroblasts. Although the mechanism of its action is not entirely clear, at least part appears to be through the ability of bradykinin to induce fibroblasts to produce prostaglandins of the E series which "feed back" to suppress collagen production. Recent studies have also demonstrated that human lung fibroblasts are heterogeneous in both the amounts of collagen they produce and their responses to growth factors. Thus, this observation leads to the concept that the expansion of high producing fibroblast clones rather than low producing clones may explain, in part, why some individuals are more "susceptible" to fibrosis than others, i.e., some individuals have a predominance of fibroblasts that are high producers of collagen and hence are more likely to repair lung injury with an excess of scar tissue.

Although the alveolitis of the interstitial lung disease is the major mechanism modulating injury and repair, certain interstitial lung diseases are known to be initiated by direct injury to the parenchymal cells. Included in such a category is the disease caused by the inhalation of high concentrations of oxygen for extended periods and the disease caused by paraquat, a commonly used herbicide. It is known that with inhalation of 100% oxygen for 24 hours there is a loss in lung function which is reversible if the oxygen administration is stopped. In order to understand the earliest biologic changes in the lung that precede the more permanent fibrotic form of hyperoxic lung injury, studies have been carried out with normal volunteers in which oxygen has been administered for 18 hours. These studies have demonstrated that there is a 2-3 fold increase in albumin in alveolar epithelial fluid suggesting that hyperoxia for 18 hours is associated with alveolar capillary damage and subsequent "leak." In addition, alveolar macrophages recovered from such individuals demonstrate they spontaneously release both fibronectin and the alveolar macrophage derived growth factor. Although these changes are reversible if the oxygen is stopped, they suggest possible mechanisms by which these individuals develop subsequent fibrosis. It has been known for some time that ingestion of paraquat either accidently or by suicidal intent leads to progressive pulmonary fibrosis and often death within several days to weeks. Recent observations, however, have suggested that paraquat can also be absorbed through the skin and this may be an alternative route for paraquat exposure. Interestingly, although paraquat is known to be cytotoxic to lung parenchymal cells when directly applied to animal or human parenchymal cells in vitro studies with animals have demonstrated that, following paraquat administration, there is also an alveolitis that rapidly develops characterized by an increased number of macrophages and neutrophils in the alveolar structures. Furthermore, the macrophages in paraquat exposed animals are spontaneously releasing fibronectin and there is increased amounts of fibronectin in the alveolar epithelial fluid. In addition, the macrophages are releasing the growth factor for fibroblasts suggesting this may be one mechanism by which rapid fibrosis occurs following entrance of paraquat to the body.

Morphologic evaluation of epithelial cells from lung biopsies from patients with a variety of interstitial lung diseases has shown that there are striking changes to the epithelial cell population. There is a loss of alveolar type I cells and replacement with alveolar type II cells together with bronchial epithelial cells that "grow down" into the alveoli to replace the injured type I cells. Furthermore, there is a piling up of epithelial cells to give the appearance of metaplasia.

Evaluation of patients with histiocytosis-X, an interstitial lung disease of young adults associated with wide spread cystic disease has shown that there are markedly increased numbers of Langerhans cells comprising the alveolitis. While such cells, known derivatives of the mononuclear phagocyte system, are present in the alveolar structures in other interstitial lung diseases, histiocytosis-X is the only disease in which these cells are found in the bronchoalveolar lavage. In the past, these cells could only be identified by electron microscopy but recent studies have shown that they have a cell surface antigen identified by OKT6 monoclonal antibodies. This may prove a useful indicator of disease activity in such patients. This is an important concept because, to date, no other roentenographic, physiologic, scintigraphic or lavage parameters have been found that can accurately predict which patients will deteriorate and which will not.

Significance to Biomedical Research and the Program of the Institute: The interstitial lung disorders and destructive lung disorders are almost uniformly fatal and affect a significant proportion of the population. There has been little information on the natural history, etiology, pathogenesis, pathophysiology and therapy of these disorders. By combining studies of patients with these disorders with our basic research program concerning basic immunology as well as evaluation of the control of synthesis and degradation of the of the extracellular matrix, we expect to make major inroads into understanding and treating these disorders.

Proposed Course: Studies as outlined will be continued. As methods are developed in the basic laboratory, they will be applied to study the biopsy specimens from human lung. Particularly important are the studies in lung explants and tissue culture where the manipulation and control of connective tissue synthesis and degradation can be explored using various pharmacologic agents. Immunologic studies will continue to explore cell-mediated mechanisms in these disorders. As the result of pharmacologic agents become promising, they will be studied in patients where applicable.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02407-08 PB

PERIOD COVERED
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Destructive Lung Disease

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The destructive lung disorders are diseases in which there is a desolution and loss of alveolar structures. The primary mechanism responsible for this is an imbalance of proteases and antiproteases toward proteases, particularly neutrophil elastase. Studies of patients with hereditary PiZ homozygous α1-antitrypsin deficiency demonstrated that they have neutrophils in their lungs, active neutrophil elastase and very little α-1 antitrypsin. Intraveneous therapy with partially purified α1-antitrypsin to these individuals demonstrate not only is the antielastase screen of the lower respiratory tract restored but the active elastase is removed. Although there are some patients with this hereditary disorder that do not have emphysema, the primary determinant of disease appears to be the presence of neutrophils in the lung as well as the neutrophil proteolytic enzymes. Therapy with Danazol, and impeded androgen, has shown that serum α1-antitrypsin levels can be maintained 50 percent above baseline levels for extended periods of time without complications.

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The Destructive Lung Disease. The destructive lung disorders are characterized by loss of the alveolar structures. The current concepts of the pathogenesis of these disorders are defined by the so called "protease-antiprotease" theory of emphysema. This theory holds that in normal lung, proteases (e.g., elastase) released by inflammatory and immune effector cells within the alveolar structures are balanced by antiproteases (e.g., α1-antitrypsin, a circulating antiprotease that diffuses into the lung). In the destructive lung diseases there is increasing evidence that there is an imbalance such that the proteases overpower the anti-protease systems, thus causing connective tissue destruction and loss of the alveolar structures.

The interest of the Pulmonary Branch in the destructive lung diseases has been two fold: (1) evaluating the protease-antiprotease balance in the lower respiratory tract of humans; and (2) since elastin is a primary connective tissue target in destructive lung disease, studying elastin biosynthesis and the role of elastin fragments in the pathogenesis of these disorders. The major clinical group of patients studied in relation to destructive lung diseases is the hereditary disease α1-antitrypsin deficiency. In individuals who are homozygous for this disease, emphysema develops in the fifth decade and usually is fatal.

Previous studies from our laboratory have demonstrated that patients with PiZ, α1-antitrypsin deficiency are entirely devoid of protection against neutrophil elastase in the lower respiratory tract. In addition, studies have demonstrated that the infusion of partially purified α1-antitrypsin intravenously on a once a week basis results in the reestablishment of the antielastase protection in the alveolar structures of these individuals. Studies in the past year have demonstrated that patients with α1-antitrypsin deficiency can be divided into two groups. The first group includes individuals with clear manifestations of destructive lung disease while the second group includes those who appear to have relatively normal lungs. Evaluation of the determinants of disease expression in the PiZ individuals show that although the two groups have similar α1-antitrypsin levels in blood and lung and have similar amounts of elastase per neutropil, the group of individuals who have destructive lung disease have evidence of neutrophils in the lung, active neutrophil elastase in their alveolar epithelial fluid, and alveolar macrophages which are spontaneously releasing neutrophil chemotactic factor. In addition to neutrophil elastase, these individuals also have evidence of neutrophil myeloperoxidase in their alveolar epithelial fluid. Since such patients also have evidence of loss of alveolar parenchymal cells, the presence of myeloperoxidase may be one mechanism (i.e., oxidant mediated) by which such cells are destroyed.

Since α1-antitrypsin deficiency is a single point mutation disease involving the structure of the α1-antitrypsin molecule, it is unclear why the alveolar macrophages of these individuals are spontaneously releasing neutrophil chemotactic factor. However, in vitro studies of normal alveolar macrophages together with neutrophil elastase, neutrophil collagenase, α1-antitrypsin, α2-macroglobulin, α2-macroglobulin-elastase complex, α2-macroglobulin-collagenase complex, and α1-antitrypsin-elastase complex have demonstrated that the presence of proteases in the alveolar structures may be related to the stimulation of release of neutrophil chemotactic factor from the alveolar macrophages. In this context, neutrophil elastase stimulates human alveolar macrophages to release the neutrophil chemotatic factor. However, collagenase does not stimulate chemotactic factor release nor does α1-antitrypsin alone nor α2-macroglobulin

alone. Interestingly, neutrophil elastase complexed with α1-antitrypsin will not stimulate the macrophages to release neutrophil chemotactic factor suggesting that if there are sufficient amounts of α1-antitrypsin in the alveolar structures (i.e., in the PiZ individual resulting from α1-antitrypsin replacement therapy) the macrophages would not be stimulated to release the neutrophil chemotatic factor and hence would not be attracting neutrophils to the lung. Interestingly, in vitro studies have demonstrated that elastin fragments are chemotactic for blood monocytes providing a mechanism by which the alveolitis of a disease like α1-antitrypsin deficiency (or the emphysema associated with cigarette smoking) may be maintained.

Studies with partially purified human α1-antitrypsin have demonstrated that the anti-elastase protection of the alveolar structures of PiZ individuals can be reestablished with once a week infusions of 4 grams of α1-antitrypsin. Recent evaluation of the alveolar epithelial fluid of these individuals has shown that: before the infusion active neutrophil elastase present in their alveolar structures; two days after the second week of infusion and two days after the fourth week of infusion neutrophil elastase is no longer detectable. Thus, intraveneous α1-antitrypsin not only reestablishes the balance of antiproteases in the lower respiratory tract but also inhibits the active proteases from further destruction of the alveolar structures.

Studies have continued on the use of Danazol, an impeded androgen, which has been previously shown to increase the serum α1-antitrypsin levels in PiZ homozygous individuals to approximately 50 percent above baseline levels. Several patients have now been maintained on chronic Danazol therapy and have maintained their elevated α1-antitrypsin serum levels with very little in the way of serious side effects.

Physiologic comparisons of PiZ α1-antitrypsin deficient individuals with patients of idiopathic pulmonary fibrosis demonstrate that they have remarkable similarities in exercise-induced abnormalities even though they have very disparate diseases. The common denominator appears to be the loss of the alveolar structures. Both sets of patients significantly decrease arterial PaO_2, with exercise while maintaining constant $PaCo_2$ levels and both groups have similar maximal oxygen consumptions (both very low compared to normal individuals of the same age).

Although the neutrophil is clearly the major source of elastase in the alveolar structures of individuals who have an accumulation of that effector cell in the lower respiratory tract (i.e., α1-antitrypsin deficiency, idiopathic pulmonary fibrosis) there has been interest in the alveolar macrophage as a possible source of proteolytic enzymes in the lung. However, although animal alveolar macrophgaes release significant amounts of collagenase and elastase, human alveolar macrophages release very little and do so only after several days in culture. Furthermore, the release of this enzyme appears to be "constitutive", i.e., it cannot be stimulated by phagocytosis, immune complexes or complement components. Thus, the production of elastase by the alveolar macrophage probably plays little role in the emphysematous process except in the cases when there are markedly increased numbers of macrophages. It is important to recognize, however, that the macrophage elastase is a very different enzyme than the neutrophil enzyme. While the latter is a serine-protease, the former is a metalo-enzyme, a very unusual form of elastase.

The tight-skin mouse represents a hereditary disorder of emphysema. Physiologic studies of these animals have demonstrated that they have classic findings of emphysema and morphologic studies of their alveolar structures demonstrate clear evidence of destruction. The mechanisms of destruction are unkonwn but recent studies evaluating inflammatory and immune effector cells in the lower respiratory tract of these animals have shown that they have neutrophils whereas normals animals do not.

Major strides have been made in the development of technology to evaluate elastin gene biosynthesis. A fragment of an elastin gene has been isolated from the sheep genome. This gene has a structure similar to other genes (i.e., introns and exons) and exons are being isolated for use as probes to determine elastin gene number and gene localization. In addition, complementary DNA has been synthesized from isolated elastin mRNA and used to quantitate elastin mRNA levels in developing lung. Consistent with morphologic studies, biochemical studies indicate that the elastin levels in the developing sheep fetal lung continue to increase. This is paralleled by increased rates of biosynthesis by the tissue and by increases in the elastin mRNA levels, suggesting elastin control at the transcriptional level.

Significance to Biomedical Research and the Program of the Institute: Destructive lung diseases are usualy fatal and cause a significant amount of morbidity in our population. Studies of the pathogenesis of these disorders have elucidated the mechanism of the lung destruction and therapeutic approaches show promise in terms of preventing these processes.

Proposed Course: Studies as outlined will be continued and the mechanisms of proteases and antiproteases in the lower respiratory tract evaluated in more detail. In addition, studies are being instituted to evaluate oxidant mechanisms as a cause of destruction in these disorders.

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ANNUAL REPORT OF THE

CLINIC OF SURGERY

NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

October 1, 1981 through September 30, 1982

The clinical and laboratory programs of the Surgery Branch have, as in past years, largely centered upon the study of the operative methods for the correction of congenital and acquired heart and lung diseases, assessment of the results of such operations, and laboratory studies related to cardiovascular physiology and pharmacology.

Operations for Left Ventricular Outflow Tract Obstruction and Coronary Artery Bypass Graft or Valve Replacement. Nine patients have undergone combined operation for left ventriculomyotomy and myectomy and coronary artery bypass grafting. There are 8 men and one woman in the group and their ages at the time of operation ranged from 42 to 64 years. Three had a single bypass graft, 4 had double bypass and 2 had triple grafts (17 grafts total). There was one early postop death from mediastinitis. Four patients have been operated upon since September 1981 and followup is not yet available. Seven patients have undergone combined operation for left ventriculomyotomy and myectomy and valve replacement. Three patients had mitral and four had aortic valves replaced. Two men and one woman had mitral valve replacement (ages 18 to 46 years) and three women and one man had aortic valve replacement (ages 51 to 64 years).

Combined left ventriculomyotomy and myectomy and coronary artery bypass grafts: Three patients had a single coronary artery bypass, four had double bypass and two had triple bypass (17 grafts total). There was one early postop death (less than 30 days) from mediastinitis. Four patients have been operated on since September 1981 and follow up is not yet available. Follow up ranging from 1.5 to 6.5 years is available on four patients with eight grafts (one single, two double, one triple). All four patients had good relief of obstruction and five of the eight grafts were patent at the time of postop catheterization. Two of these patients are alive and NYHA functional class II 1.5 and 3 years after operation. One of the patients had undergone a single bypass and the graft was occluded at the time of postop catheterization. He has some residual angina. The other patient underwent triple coronary artery bypass graft procedure and all grafts were patent. This patient has had no further angina. The other two patients died 2 and 6.5 years after myotomy and myectomy and double coronary artery bypass grafting. In each of these patients one graft was patent at the time of postop study. Both patients died suddenly. The patient surviving 2 years was hospitalized at the time of death for evaluation of metastatic lung cancer and was found dead in his room. No necropsy was performed but was known to have had congestive heart failure symptoms and arrhythmias prior to his final illness. The other patient died suddenly at home and had suffered recurrent angina for about 4 years prior to his death. At necropsy both grafts were occluded.

Coronary artery disease after left ventriculomyotomy & myectomy: Two patients who underwent only LVM&M returned late postop for evaluation of angina and were found to have significant coronary artery disease. One of these patients underwent CABG ten years after myotomy & myectomy. He died intra-operatively. The other patient presented 8.5 years post myotomy and myectomy and he had a PTCA performed.

Combined left ventriculomyotomy and myectomy and valve replacement: Mitral Valve: There were two men and one woman in this group and their ages at the time of operation ranged from 18 to 46 years. All three had mitral regurgitation. One patient had ruptured chordae; one had a congenital deformity of the mitral valve; the third patient had a grossly normal appearing valve which, however, was significantly regurgitant and had to be replaced before the patient could be weaned from bypass. There was one perioperative death. A second patient died 4 years postop in congestive failure. A catheterization obtained shortly before her death was consistent with congestive cardiomyopathy. The third patient is alive and NYHA functional class II seven years postoperative.

Aortic Valve: There are 3 women and one man in this group and their ages at the time of operation ranged from 51 to 64 years. All four patients had aortic regurgitation on preop catheterization and two of the four had a history of endocarditis. At operation one patient with previously treated endocarditis was found to have a perforation of one of the aortic valve leaflets. The second patient with a history of endocarditis had a grossly retracted valve. The third patient had a bicuspid calcified valve and the fourth patient was found to have AV leaflets that were thickened, shortened, and contained some calcium. All four patients in this group survived operation. Three of the four underwent postop catheterization and were found to have good relief of their outflow obstruction.

Bioprosthetic Valvular Failure. Clinical and Pathological Characteristics in an Experimental Animal Model. The purpose of this project was to develop an animal model to assess the pathologic alterations and the hemodynamic dysfunction resulting from those alterations of cardiac bioprosthetic valves. Bioprosthetic valves implanted in juvenile sheep demonstrate the same pathologic alterations of degeneration and calcification as those implanted in humans, but these alterations occur in a much shorter period of time in valves implanted in sheep. Bioprosthetic valves of clinical quality from all commercial sources in this country have been implanted in the tricuspid and mitral positions of sheep to evaluate the pathogenesis of bioprosthetic valvular degeneration and to compare the characteristics and severity of these alterations in the different types of bioprosthetic valves.

The development of an ideal cardiac valve substitute remains a major problem in cardiac surgery. Bioprosthetic cardiac valves have become the valves of choice at many institutions, primarily because they do not require chronic anticoagulant therapy. However, it has become apparent that the long-term durability of bioprosthetic valves is finite; degeneration and calcification of these valves have become major complications of long-term implantation.

An in vivo investigational model is needed for the study of the mechanisms of degeneration and calcification in substitute bioprosthetic cardiac valves. For this purpose we have met the following criteria in an investigational model developed in domestic sheep (Ovis aries): 1) the bioprostheses should be implanted in growing animals to simulate the physiologic conditions in young humans, in whom accelerated degenerative alterations of bioprosthetic valves are known to occur; 2) the pathologic alterations should develop within several months after implantation to permit expeditious study; 3) the animals must be of suitable sizes for standard cardiopulmonary bypass techniques; 4) at maturity the animals should not outgrow the bioprosthetic cardiac valves; and 5) the pathologic alterations in the bioprosthetic valves must be similar in the animals and in humans. We

have implanted 157 bioprosthetic cardiac valves in juvenile domestic sheep. Of these 157 valves, 23 were explanted early (<one month), 76 were explanted late (>one month - mean 4.2 ± 1.7 months) and 58 remain implanted. Clinical, hemodynamic and/or morphologic deterioration occurred in all 76 BPV. Structural alterations included calcific deposits (74/76), fibrous sheaths upon the leaflets (71/76), intracuspal hematomas (5/76) and infection (12/76). Of 45 sheep having satisfactory early and late hemodynamic studies, all had BPV with increased mean or end-diastolic gradients and/or decreased valve areas. Calcium content in tricuspid valves = 196 ± 44 mg/g; in mitral valves = 96 ± 14 mg/g; and in unimplanted valves = 1.2 ± 0.2 mg/g tissue dry weight. All of the above findings are similar to those in BPV removed from humans. Thus, implanting BPV in sheep provides an excellent means for investigating the preparation and design of the BPV.

Comparison of Dopamine and Dobutamine in the Early Postoperative Management of Patients Undergoing Mitral Valve Replacement. Low cardiac output syndrome is a common clinical problem early after mitral valve replacement. In addition, these patients often have increased pulmonary vascular resistance (PVR) which may be exacerbated by commonly used inotropic agents. This study compared the hemodynamic effects of dopamine and dobutamine, over a range of therapeutic doses, in 10 patients within several hours after mitral valve replacement. Both dopamine and dobutamine are effective inotropic agents, producing progressively increasing cardiac output and arterial pressure with increasing drug dosages. Both agents also cause moderate tachycardia and we did not demonstrate any difference in this effect between the two drugs. While dobutamine had no effect on PVR, there was a significant decrease in PVR with dopamine infusion.

Ten patients who underwent mitral valve replacement were studied on the first afternoon after operation. While low cardiac output was not a contraindication to study, patients who were dependent on pressors other than dopamine or dobutamine were excluded from study. Hemodynamic monitoring was performed as per the standard routine for these patients. All patients had an arterial catheter for continuous monitoring of arterial pressure and a Swan-Ganz catheter for monitoring of right atrial and pulmonary artery pressures. Cardiac outputs were determined by thermodilution technique via the Swan-Ganz catheter. Left atrial pressure was monitored directly via indwelling left atrial catheters. Systemic vascular and pulmonary vascular resistance were determined by standard formulas.

After a 30 minute control period, drug infusion was begun. The infusion drug was infused at doses of 2, 4, 6, 8, and 10 ug/kg/min, for 5 minutes at each dose. All hemodynamic parameters were measured at the end of each 5 minute interval. There was a second 30 minute control period between drugs, and the order of drug infusion was alternated randomly.

Both dopamine (P) and dobutamine (B) produced significant increases in cardiac index 1.68 ± 0.26 to 2.44 ± 0.38 L/min for P, and 1.61 ± 0.23 to 2.51 ± 0.38 L/min for B (control vs peak effect). Both drugs produced slight, but insignificant, increase in LA pressure and slight decrease in RA pressure ($p < 0.05$ only at 10 mg/kg/min). Mean arterial pressure was significantly increased by both drugs 84.7 ± 3.7 to 105.1 ± 4.9 mm Hg for P, and 85.1 ± 3.9 to 102.2 ± 5.5 mm Hg for B (control vs peak effect). Pulmonary artery mean pressure (PAM) was significantly increased by both dopamine and dobutamine, although dobutamine increased PAM at lower dosages. Despite claims that dobutamine is less chronotropic than dopamine, we found no difference between the drugs in this regard.

Heart rate increased from 87.6 ± 9.1 to 119.2 ± 10.6 beats/min with dopamine and from 90.4 ± 8.9 to 128.1 ± 10.9 beats/min with dobutamine (control vs peak effect). Pulmonary vascular resistance (PVR) was not significantly changed by dobutamine (418 ± 150 control dynes-cm.sec^{-5} vs 395 ± 97 minimum value). Dopamine, however, produced significant decreases in PVR (408 ± 91 dynes-cm.sec^{-5} control vs 308 ± 78 minimum value). Systemic vascular resistance (SVR) was significantly decreased by both drugs (2502 ± 377 dynes-sec.cm^{-5} to 2131 ± 285 for dopamine, and 2527 ± 335 to 2120 ± 344 for dobutamine). Thus, the only demonstrable hemodynamic differences between P and B in the present study was in their effects upon PVR.

The Influence of Minoxidil on Regional Myocardial Blood Flow and Cardiac Pathology in Beagle Dogs. The influence of minoxidil on cardiovascular hemodynamics and on regional myocardial blood flow in beagle dogs was investigated. Minoxidil caused increases in mean right atrial and left ventricular end-diastolic pressures. Systemic and pulmonary vascular resistances were reduced; cardiac outputs were increased. Left ventricular stroke work and the tension time index were unchanged by minoxidil administration. Regional myocardial blood flow was measured using radioactive microspheres. Minoxidil increased coronary blood flow to all regions of the heart except to the left ventricular papillary muscles, in which, it was unchanged in comparison to blood flow in untreated animals. Minoxidil increased blood flow to left ventricular subendocardial tissue but significantly less than corresponding areas of subepicardial tissue, reducing the subendocardial/subepicardial blood flow ratio.

Ten adult beagle dogs, weighing between 8 and 12 kg, were given two doses of minoxidil (3 mg/kg) 24 hours apart. Shortly thereafter they were anesthetized with alpha chloralose (60 mg/kg, intravenously) and the heart exposed through a left thoracotomy. Aortic, left atrial and left ventricular pressures were recorded. Cardiac output was measured by the thermal dilution technique utilizing a flow directed catheter inserted through an internal jugular vein and positioned in the pulmonary artery. Regional myocardial blood flow was measured using radioactive microspheres (^{46}Sc) 9 ± 1 u in diameter. At the conclusion of the study the heart was arrested with potassium chloride, excised, washed and placed in 10% buffered formalin for three days. The heart was then separated into atria and ventricles. The epicardial fat and epicardial coronary vessels were discarded. The atria were divided into right, left and septal portions. The ventricles were divided into right ventricular outflow and inflow (body) portions, right side of ventricular septum, left side of ventricular septum, anterior and posterior portions of left ventricular free wall, and anterior and posterior papillary muscles of the left ventricle. The ventricular free walls were further subdivided into subendocardial and subepicardial layers of equal thickensses. Each of the above regions will undergo complete histologic evaluation.

A second group of ten beagle dogs were given a placebo instead of minoxidil. These animals served as controls and were used for comparison with the minoxidil treated group. Results are expressed as mean values ± standard errors of the means.

Blood Flow (cc/min/gm)

	Ant. Pap. Muscle	Post. Pap. Muscle
Control	1.90 ± .21	1.85 ± .18
Minoxidil	2.32 ± .41	2.21 ± .25

Blood Flow (cc/min/gm)

Anterior

	LV Endocardium	LV Epicardium	Endo/Epi
Control	1.41 ± .06	1.46 ± .06	0.91 ± .03
Minoxidil	**2.23 ± .13	**2.91 ± .15	**0.77 ± .04

Posterior

	LV Endocardium	LV Epicardium	Endo/Epi
Control	1.54 ± .08	1.50 ± .08	1.03 ± .02
Minoxidil	**2.72 ± .13	3.48 ± .15	**0.79 ± .06

Hemodynamic Parameters

	HR beats/min	CO cc/min/kg	AO mm Hg	LV mm Hg
Control	134 ± 5	230 ± 20	125/88 ± 4/3	118/5 ± 5/1
Minoxidil	**152 ± 14	**340 ± 20	**118/60 ± 7/5	**103/14 ± 7/1

	SVR dyne sec cm^{-5}	PVR dyne sec cm^{-5}	LVSW gm m/kg/beat	TTI mm Hg sec/min
Control	3272 ± 287	307 ± 17	2.34 ± 0.2	960 ± 74
Minoxidil	**1376 ± 156	**143 ± 20	1.91 ± 0.13	980 ± 100

** $P \leq 0.05$

Temporary Relief of Pulmonary Regurgitation with a Balloon "Valve". In the course of surgical correction of several forms of congenital heart disease, the pulmonary valve may be rendered regurgitant. Over the long term this is very well tolerated. In the acute postoperative period, however, right ventricular failure is a significant and potentially life threatening complication. While the cause of RV failure may be multifactorial, the extra work produced by the pulmonary regurgitation may worsen the RV failure. This study evaluated in laboratory animals the use of a balloon device to occlude the pulmonary artery during diastole and thus prevent pulmonary regurgitation. The balloon is connected to the control panel for an intra-aortic balloon pump to control the inflation and deflation of the balloon. In 8 sheep the pulmonary valve was completely excised. This resulted in pulmonary regurgitation which was compensated by increased stroke volume and greatly increased RV stroke work. Use of the pulmonary artery balloon, functioning as a "valve", completely prevented regurgitaton and reduced RV work.

Pulmonary regurgitation was produced in 8 sheep by complete excision of the pulmonary valve. This produced an increased forward stroke volume to compensate for the 40% regurgitant fraction. The pulmonary artery pulse pressure was increased, primarily by a drop in the PA diastolic pressure. The pulmonary flow pattern was monitored via an electromagnetic flow probe on the main PA. The forward and backward (i.e. regurgitant) flow was calculated by planimetry of the area under the flow curve above and below the zero level. The use of a balloon "valve" to occlude the pulmonary artery during diastole resulted in complete elimination of pulmonary regurgitation. The PA pulse pressure was decreased from an average of 22.7 to 11 mm Hg when the 'valve' was functioning. Use of the valve decreased the stroke volume from an average of 47 to 26 cc/beat and decreased the stroke work from an average of 11 to 5.25 gm M/beat.

960

The use of a simple, inflatable balloon to occlude the pulmonary root, controlled by readily available equipment for intra-aortic balloon pumping, provides a method for temporary relief of pulmonary regurgitation. This greatly reduces RV work and hence may be of help to reverse RV failure. With time the RV should recover and then tolerate the regurgitation over a long time. This temporary "valve" device could avoid the need for implantation of permanent prosthetic valves with the known complications they carry in children.

961

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02684-04 SU

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Diastolic retroperfusion of acutely ischemic myocardium utilizing a balloon tipped coronary vein catheter

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Steven R. Gundry, M.D., Clinical Associate, Surgery Branch, NHLBI

Other: Michael Jones, M.D., Senior Surgeon, Surgery Branch, NHLBI
Harry Seipp, Chief Technician, Surgery Branch, NHLBI
Rodger E. Solomon, Electrical Engineer, DRS

COOPERATING UNITS (if any)
Biomedical Engineering, DRS

LAB/BRANCH
Surgery Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung & Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3-1/2	2-1/2	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Despite advances in intra-aortic balloon pumping and emergency myocardial revascularization, salvage of ischemic myocardium has been limited in man by the lack of effective collateral circulation into an area of acute infarction. We are currently carrying out experimental trials of perfusing ischemic myocardium by retrograde diastolic pulsation of oxygenated blood into the coronary veins draining an area of ischemia via a balloon tipped catheter that can be introduced transvenously. Results to date indicate correction of EKG changes, reversal of dyskinetic areas and improvement in myocardial performance in acutely ischemic myocardium following institution of retroperfusion.

962

Description: A double lumen balloon-tipped catheter is placed retrogradely into the coronary sinus in an experimental animal and advanced into the left anterior descending coronary vein to an extent that when the balloon is partially inflated the lumen is obstructed, but unobstructed to normal systolic venous flow when deflated.

This catheter is connected to an oxygenated blood source, the brachial artery through a synchronized 20 cc pulsatile assist device which is driven by a standard electrocardiogram-actuated intra-aortic balloon counterpulsation pump. After creating an area of ischemic myocardium by LAD ligation, and recording myocardial dynamics with flow transducers and intramyocardial ultrasonography, the coronary vein perfusion pump is actuated. Oxygenated blood in injected retrograde into the coronary vein in diastole via the balloon catheter, whose balloon simultaneously fills and obstructs venous drainage during the injection. The pump is then deactivated during systole. The balloon deflates, and blood drains from the coronary vein into the right atrium in the normal fashion. Myocardial dynamics are reassessed during this procedure.

We designed and experimentally evaluated a transvenously introduced double-lumen balloon tipped catheter and ECG activated pumping system for perfusing ischemic myocardium by retrograde pulsation of oxygenated blood into the coronary veins during diastole. Balloon deflation during systole allowed normal venous drainage. Sixteen dogs were instrumented with sonomicrometry crystals and catheters to measure regional and global left ventricular (LV) function. The left anterior descending coronary artery (LAD) was occluded for 40 minutes. Following 10 minutes of ischemia, the affected LV regions were dilated and dyskinetic. Coronary vein retroperfusion (CVRP) was instituted for 30 minutes in 8 dogs (controls were not perfused). CVRP restored 37% of LV systolic shortening (1.5 mm vs 0.6 mm)*; controls had no systolic shortening. Regional LV dilation of 2.2 mm was reduced to 1.5 mm by CVRP; controls expanded to 3.1 mm*. Endocardial ST segments returned to normal with CVRP, but elevated 19 mm in controls*. Cardiac output rose to baseline levels with CVRP but remained 1.5 L/min depressed in controls*. Three of 8 controls died. Four of 5 remaining dogs died from LV fibrillation after LAD release; all CVRP dogs recovered. CVRP offers a transvenous approach for assisting and perfusing failing, ischemic myocardium.*
*($p < 0.001$)

Proposed Course: The study has been completed

Publications:

Gundry, S. R., Seipp, H. W. Jr., Solomon, R.E.: Modification of myocardial ischemia in normal and hypertrophied hearts utilizing diastolic retroperfusion of the coronary veins. J. Thorac Cardiovasc Surg 83:659-669, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02686-04 SU

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Endothelial changes in human saphenous veins prepared for coronary artery bypass grafts: Effects of distention pressure and preservation techniques.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Steven R. Gundry, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Michael Jones, M.D., Senior Surgeon, Clinic of Surgery, NHLBI

OTHER: Victor Ferrans, M.D., PhD., Chief, Ultrastructure sec., Pathology Branch
Tokuhiro Ishihara, M.D., Guest Investigator, Ultrastructure sec., Pathology Branch

COOPERATING UNITS (if any)
Pathlogy Branch, NHLBI

LAB/BRANCH
Clinic of Surgery

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2-1/4	2-1/4	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Endothelial changes in saphenous veins harvested for coronary artery bypass grafts were found when veins prepared in this manner were examined using the scanning and transmission electron microscopes. Using current distention pressures and preservation techniques, severe endothelial disruption was found. We compared the endothelial changes caused by a variety of currently employed vein handling techniques to determine the best method to protect venous endothelium during the bypass procedure.

964

PHS-6040
(Rev. 2-81)

DESCRIPTION: The saphenous vein has become the conduit of choice in coronary artery revascularization procedures. Despite its popularity and usefulness, clinical and pathological studies indicate that these vein grafts are subject to early closure and mild to severe intimal hyperplasia or atherosclerosis. Experimental models have demonstrated that endothelial damage or disruption can predispose to these conditions. To evaluate the endothelium of saphenous veins prepared in the usual manner at this Institute and others, a portion of the harvested vein taken for coronary artery bypass grafts in patients was immediately fixed in a distended condition at mean arterial pressure for electron microscopy. These veins were compared to veins which were distended and fixed at venous pressure alone.

Endothelial damage occurring during preparation of saphenous vein grafts is an important cause of early and late graft failure. To determine optimal preparation techniques for human saphenous veins (SV) we made a scanning electron microscopic comparison of the effects of handling techniques, immersion media and distention pressures on human SV morphology. Segments of SV from 30 patients were divided into 7 groups of 5 veins each. Group 1 was immediately distended and fixed with glutaraldehyde at 100 mm Hg. Groups 2 and 3 were immersed in normal saline for one hour at 4°C and 28°C, respectively, and then distended to 100 mm Hg with saline. Groups 4 and 5 were immersed in blood for one hour at 4°C and 28°C, respectively, followed by distention to 100 mm Hg with blood. Groups 6 and 7 were immediately distended with saline group (6) or blood group (7) to 300 mm Hg. One SV from each group was grasped with a vascular clamp; one SV in each group also contained a side branch which had been ligated. All groups were fixed by perfusion with glutaraldehyde at 100 mm Hg to simulate arterial pressure and examined with a scanning electron microscope. SV immersed in warm saline sustained massive endothelial cell loss, while SV immersed in warm blood showed only moderate damage. Cold blood and cold saline immersion fully preserved endothelium; however, saline immersion produced mural edema. Distention to 300 mm Hg with saline produced severe endothelial damage and edema, an effect lessened by blood distention. Vascular clamping destroyed endothelium and fractured the intima. Marked luminal stenoses were caused by 4 of 7 side branch ties that appeared normal externally. We conclude that human SV are best preserved by a "no touch" harvesting technique, minimizing manipulation, placement of side branch ties away from the SV wall, immersion in cold blood, and avoidance of distention above 100 mm Hg.

RESULTS: The project has been completed.

PUBLICATIONS: Jones M, Gundry SR, Ishihara T, Ferrans VJ: Reply to letter to the Editor: Intraoperative trauma to human saphenous veins: Scanning electron microscopic comparison of preparation techniques. Ann Thorac Surg 33:100, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 02697-03 SU

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Characterization of naturally occurring, genetically transmitted, fibrous subaortic stenosis in Newfoundland dogs.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Michael Jones, M.D., Senior Surgeon & Investigator, Clinic of Surgery, NHLBI

OTHER: Anthony L. Picone, M.D., Clinical Associate, Clinic of Surgery, NHLBI
A. Michael Borkon, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Victor J. Ferrans, M.D., Ph.D., Chief, Ultrastructure Sec. Pathology Br.
William C. Roberts, M.D., Chief, Pathology Branch, NHLBI
Joseph E. Pierce, DVM, Chief, Sec. Laboratory Animal Medicine & Surgery, NHLBI

COOPERATING UNITS (if any)
Pathology Branch and Section of Lab Animal Medicine & Surgery, NHLBI

LAB/BRANCH
Clinic of Surgery

SECTION

INSTITUTE AND LOCATION
National Heart, Lung and Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	3	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have been characterizing the morphologic and the hemodynamic abnormalities occurring in Newfoundland dogs with genetically transmitted subaortic stenosis. Of 176 animals studied thus far, almost one-half have hemodynamic and/or morphologic evidence for left ventricular outflow tract obstruction. The obstruction is absent at birth, appearing after one month of age. The lesion presents clinically by the presence of a precordial murmur, thrill, arrhythmia, congestive heart failure, bacterial endocarditis of the aortic valve, or sudden death. Obstruction to left ventricular outflow is caused by the development of a circumferential, subaortic fibromuscular ring continuous with the anterior leaflet of the mitral valve, virtually identical to the same lesion in humans. The obstruction is associated with left ventricular hypertrophy, intramural coronary artery lesions, myocardial fibrosis, and abnormalities of myocardial blood flow.

966

PHS-6040
(Rev. 2-81)

Description: In the 176 dogs studied hemodynamic and/or morphologic evidence of LV outflow tract obstruction (LVOTO) was present in 66 animals, including 25% of 77 1 year old, 38% of 76 aged 13 - 24 months, 73% of 30 aged over 24 months. None of 22 newborns and 1 of 5 1 month old had LVOTO. No dogs had asymmetrical septal hypertrophy or myocardial cellular disarray. LVOTO was associated with LV hypertrophy (LV/body weights 4.5 g/kg), sudden death (n=16), thickened aortic valves, bacterial endocarditis (n=9), intramural coronary artery lesions, myocardial fibrosis and abnormalities of myocardial blood flow. All dogs with LVOTO had circumferential subvalvular fibrous rings continuous with the ventricular septum and anterior mitral leaflet. Of 64 dogs without LVOTO the septal endocardium was thickened in 38%. Although present in a few dogs without LVOTO, an unusual angulation of the aorta and the septum occurred in dogs with LVOTO. Subaortic stenosis and its sequelae in Newfoundland dogs appears to be acquired after birth and its pathogenesis to be due to the interaction of genetic, morphologic, and hemodynamic factors.

Publications: Borkon AM, Jones M, Bell JH, Pierce JE: Regional myocardial blood flow in left ventricular hypertrophy: An experimental investigation in Newfoundland dogs with subaortic stenosis. J. Thorac. Cardiovasc. Surg. In Press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02698-03 SU

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Postoperative cardiac care of infants and children

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Michael Jones, M. D., Senior Surgeon, Surgery Branch, NHLBI
Lily Ng, R.N., M.S.N., Supervisor Clinical Nurse, Clinical Center

COOPERATING UNITS (if any)

Nursing Department, The Clinical Center

LAB/BRANCH

Surgery Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung & Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3/4	3/4	

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cardiac surgery in infants and children has progressed so that most lesions may be corrected early in life. In theory and in practice the postoperative management of infants and children is similar to that of adult patients. Nevertheless, the potential for growth, the particular and often immature metabolic prosesses, and the cardiovascular physiology of congenital malformations demand special postoperative considerations. From our experiences in the Surgery Branch of the NHLBI and those in the Hospital for Sick Children, London, England, we have developed guidelines for postoperative management to assist nurses and clinical associates with care of young patients after cardiac operations.

968

Description: The special problems of postoperative cardiac care in infants and children have been divided into the following categories: 1) preoperative considerations; 2) choice of operative procedure; 3) initial postoperative assessment; 4) basic procedures; 5) cardiovascular problems, including heart rate, preload, afterload, contractility, subacute and chronic heart failure, and rhythm disturbances; 6) respiratory problems, including care of infants and children on ventilators, weaning from ventilatory support, and problems of phrenic nerve paralysis; 7) cardiopulmonary resuscitation; 8) fluid, electrolyte, and other metabolic problems; 9) nutrition; 10) body temperature regulation; 11) renal problems, including peritoneal dialysis; 12 hematological problems; 13) jaundice; 14) infection; 15) neurological problems; 16) medication dosages; 17) prognoses without and following operation; and, 18) parental counseling.

From the above delineated considerations we have developed postoperative management guidelines, particularly directed toward the initiate and limitedly experienced nurse and clinical associate assisting with the postoperative care of pediatric cardiac surgical patients.

Proposed course: The postoperative management protocols for our unit are in the process of being refined. A publication specifically concentrating upon nursing considerations has been prepared.

Publications:

Jones, M. and Stark, J.: Special problems of postoperative care in infancy and childhood. In: Postoperative Cardiac Care. (Brainbridge, M.V., Ed): 220-227, Blackwell Scientific Publications, Oxford, England, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02699-03 SU

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Evaluation of operative treatment for discrete subaortic stenosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Robert D. Moses, M.D., Clinical Associate, Surgery Branch, NHLBI
Glenn R. Barnhart, M.D., Clinical Associate, Surgery Branch, NHLBI
OTHER: Andrew G. Morrow, M.D., Chief, Surgery Branch, NHLBI
Michael Jones, M.D., Senior Surgeon, Surgery Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Surgery Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung & Blood Institute

TOTAL MANYEARS: 2	PROFESSIONAL: 2	OTHER:

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Fifty-nine patients have undergone operation for fibrous subaortic stenosis. There were four (6.8%) early deaths. Forty-three of these patients have been followed for up to 24 years (mean 10.6) after operation; of 37 patients undergoing early postoperative cardiac catheterizations, 70% had satisfactory relief of obstruction. Of the 32 late survivors 75% are asymptomatic. Actuarial survival is 80 + 6% at ten years. However, late survival free of adverse events, including residual obstruction, significant aortic regurgitation, endocarditis, complete heart block or reoperation is 27 + 7% at ten years.

970

PHS-6040
(Rev. 2-81)

Description: Between 1956 and 1981 we performed resection and/or dilatation of the fibrous ring in 58 patients with fibrous subaortic stenosis. Nine patients had associated anomalies: PDA, VSD, RV outflow obstruction, coarctation, parachute mitral valve, and/or Ao to RV fistula. Four patients suffered perioperative deaths; 2 were during our very early experience. Excluding 16 foreign patients, late follow-up evaluations including cardiac catheterizations were performed at our institution for all 38 survivors 1 to 24 years (mean 11.7) postoperatively. Gradients preoperatively averaged 100 mm Hg (30-190); gradients at 1 to 174 months (mean 20 months) postoperative averaged 38 mm Hg (0-200). Eleven patients had residual gradients over 40 mm Hg, of whom 7 had the tunnel form of this type of LV outflow obstruction. There were 7 late cardiac deaths. Of the long-term survivors 24 of 32 are symptomatic. Nine patients have required reoperation and 3 patients have developed bacterial endocarditis. Aortic regurgitation was present in 30 patients preoperatively; 7 additional patients have it late postoperatively. Electrocardiographic criteria for LVH were present in 36 patients preoperatively and in 18 late postoperatively. Average echocardiographic LV systolic and diastolic dimensions were normal late postoperatively, 29 and 48 mm, respectively. Acturarial survival is 86% at 5 years (n=34), 80% at 10 years (n=23), and 62% at 20 years (n=6). Survival without adverse cardiac abnormalities (residual gradient greater than 50 mm Hg, significant aortic regurgitation, bacterial endocarditis, complete heart block or reoperation) is 32% at 5 years, and 27% at 10 years. We conclude that operative correction relieves symptoms and obstruction and gives satisfactory late results for most patients with fibrous subaortic stenosis, but residual cardiac abnormalities require continuing long-term follow-up.

Proposed course: The patients included in this report and additional patients as they undergo operations will be included in our continuing evaluation of the natural history of this operatively treated congenital cardiac anomaly.

Publications:

Jones, M., Barnhart, G. R., and Morrow, A. G.: Late results after operations for left ventricular outflow tract obstruction. Am. J. Cardiol. 50: In Press (August publication).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02707-03 SU

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Reperfusion with blood cardioplegia provides improved recovery of both early and late ventricular function after ischemic injury

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Daniel M. Rose, M.D., Guest Worker, Surgery Branch, NHLBI

Other: Glenn R. Barnhart, M.D., Clinical Associate, Surgery Branch, NHLBI
Jean-Paul Koch, M.D., Clinical Associate, Surgery Branch, NHLBI
Michael Jones, M.D., Senior Surgeon, Surgery Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Surgery Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung and Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1¼	1	¼

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It has been proposed that modification of the initial reperfusion solution after aortic occlusion, may alter the extent of ischemic injury. We, therefore, studied initial reperfusion with either cold blood or cold blood cardioplegia after ischemic injury. Reperfusion with cold blood cardioplegia provides better recovery of ventricular function and compliance immediately, 21, and 120 days postoperatively than cold blood alone.

PHS-6040
(Rev. 2-81)

Description and Results: We studied initial reperfusion after aortic occlusion in 15 dogs divided into three equal groups. Group I underwent 2 hours of normothermic cardiopulmonary bypass (CPB); Group II underwent 2 hours of CPB including one hour of 20°C ischemic arrest; Group III was identical to Group II but initially was reperfused with 250 cc of 25°C blood cardioplegia (BCP, K^+ = 30 mEq/L) prior to aortic unclamping. Stroke work index (SWI), LVEDP, and Vpm were compared preoperatively, immediately postoperatively, at 21 days and 120 days postoperatively. Results of SWI (GmN) are summarized below ± SEM:

	Preop	Postop	21d	120d
Gp I	36.6 ± 4.7	30.6 ± 4.2	34.8 ± 4.2	30.1 ± 2.2
Gp II	41.9 ± 3.8	16.4 ± 3.3*	28.9 ± 4.1*	27.9 ± 3.7*
Gp III	37.0 ± 2.9	22.9 ± 3.5*	31.5 ± 2.5	30.1 ± 2.3

Both Groups II and III had significant* elevations in all postoperative values for LVEDP while there were no significant* differences in the values for Vpm between Groups II and III. Hearts which were initially reperfused with BCP (Group III) had significantly* better recovery of SWI immediately postoperatively (62% vs 39%), at 21d (85% vs 69%), and at 120d (81% vs 66%) than Group II. We conclude that after ischemic injury, initial reperfusion with BCP provides better recovery of both early and late ventricular function than reperfusion with oxygenated blood alone.

*p 0.05

Course: The project has been completed.

Presentation:

Rose, D.M. Does secondary cardioplegia provide long-term recovery from ischemic injury? 18th Annual Meeting, The Society of Thoracic Surgeons, January 1982.

Publications:

Rose, D.M., Barnhart, G. R., and Jones, M.: Does secondary cardioplegia provide long-term recovery from ischemic injury? Ann Thoracic Surg: IN PRESS.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02708-03 SU

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Long-term results of repair of incomplete persistent atrioventricular canal

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Daniel M. Goldfaden, M.D., Clinical Associate, Surgery Branch, NHLBI

OTHER: Michael Jones, M.D., Senior Surgeon, Surgery Branch, NHLBI
Andrew G. Morrow, M.D., Chief, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Surgery Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung & Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Late clinical evaluation was performed on 39 patients who had repair of incomplete persistent atrioventricular canal at the NIH prior to 1976. Average follow up duration is 13 (range 5-24) years. Twenty-six patients are asymptomatic at their most recent evaluation. Actuarial survival is 88 ± 6% at 13 years after operation; survival without reoperation is 82 ± 6%. However, survival free of any late complications, including late death, reoperation, arrhythmia, or symptomatic mitral regurgitation is 52 ± 10% at 13 years.

974

PHS-6040
(Rev. 2-81)

Description: We evaluated the late results following repair of otherwise anatomically uncomplicated persistent atrioventricular canal in 39 consecutive operative survivors who underwent operation at our institution prior to 1976. Average follow-up was 12 years. Postoperative cardiac catheterization was performed in 35 patients (90%) at an average of 11 months after operation. Seven (20%) had residual mitral regurgitation with elevated mean pulmonary arterial wedge or left atrial pressures with abnormal v waves. Regurgitation was mild to moderate (PAW or LA 12-15 mm Hg) in five and severe (PAW over 20 mm Hg) in two patients. Clinically significant arrhythmias including complete heart block, sudden death, nodal rhythm and chronic atrial fibrillation occurred in seven patients (18%). Two patients have required reoperation for mitral regurgitation. Five have clinically recognizable mild to moderate mitral regurgitation controlled with medical management; twenty-five patients are asymptomatic at current evaluation. Estimated actuarial survival at 13 years is 88 $\pm$ 6%, with an actuarial survival free of reoperation of 82 $\pm$ 6%. However, actuarial survival free of any late complication including late death, reoperation, serious arrhythmia, or mitral regurgitation is only 52 $\pm$ 10% at 13 years.

Proposed course: These and additional patients as they undergo operation will be included in our continuing evaluation of the natural history of this operative treated congenital cardiac anomaly.

Publications:

Goldfaden, D. M., Jones, M., and Morrow, A. G.: Long-term results of repair of incomplete persistent atrioventricular canal. J. Thorac Cardiovasc Surg 82:669-673, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02714-02 SU

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Bioprosthetic valvular failure. Clinical and pathological characteristics in an experimental animal model.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Michael Jones, M.D., Senior Surgeon, Surgery Branch, NHLBI

Other: Glenn R. Barnhart, M.D., Clinical Associate, Surgery Branch, NHLBI
Altagracia M. Chavez, M.D., Clinical Associate, Surgery Branch, NHLBI
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Robert D. Moses, M.D., Clinical Associate, Surgery Branch, NHLBI
Tokuhiro Ishihara, M.D., PH.D., Guest Worker, Surgery Branch, NHLBI
Victor J. Ferrans, M.D., Ph.D., Chief, Ultrastructural Sec., NHLBI
William C. Roberts, M.D., Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)
Pathology Branch, NHLBI

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Surgery Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung & Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	5	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to develop an animal model to assess the pathologic alterations and the hemodynamic dysfunction resulting from those alterations of cardiac bioprosthetic valves. Bioprosthetic valves implanted in juvenile sheep demonstrate the same pathologic alterations of degeneration and calcification as those implanted in humans, but these alterations occur in a much shorter period of time in valves implanted in sheep. Bioprosthetic valves of clinical quality from all commercial sources in this country have been implanted in the tricuspid and mitral positions of sheep to evaluate the pathogenesis of bioprosthetic valvular degeneration and to compare the characteristics and severity of these alterations in the different types of bioprosthetic valves.

PHS-6040
(Rev. 2-81)

Description: The development of an ideal cardiac valve substitute remains a major problem in cardiac surgery. Bioprosthetic cardiac valves have become the valves of choice at many institutions, primarily because they do not require chronic anticoagulant therapy. However, it has become apparent that the long-term durability of bioprosthetic valves is finite; degeneration and calcification of these valves have become major complications of long-term implantation.

An in vivo investigational model is needed for the study of the mechanisms of degeneration and calcification in substitute bioprosthetic cardiac valves. For this purpose we have met the following criteria in an investigational model developed in domestic sheep (Ovis aries): 1) the bioprostheses should be implanted in growing animals to simulate the physiologic conditions in young humans, in whom accelerated degenerative alterations of bioprosthetic valves are known to occur; 2) the pathologic alterations should develop within several months after implantation to permit expeditious study; 3) the animals must be of suitable sizes for standard cardiopulmonary bypass techniques; 4) at maturity the animals should not outgrow the bioprosthetic cardiac valves; and 5) the pathologic alterations in the bioprosthetic valves must be similar in the animals and in humans. We have implanted 157 bioprosthetic cardiac valves in juvenile domestic sheep. Of these 157 valves, 23 were explanted early (<one month), 76 were explanted late (>one month - mean 4.2 $\pm$ 1.7 months) and 58 remain implanted. Clinical, hemodynamic and/or morphologic deterioration occurred in all 76 BPV. Structural alterations included calcific deposits (74/76), fibrous sheaths upon the leaflets (71/76), intracuspal hematomas (5/76) and infection (12/76). Of 45 sheep having satisfactory early and late hemodynamic studies, all had BPV with increased mean or end-diastolic gradients and/or decreased valve areas. Calcium content in tricuspid valves = 196 $\pm$ 44 mg/g; in mitral valves - 96 $\pm$ 14 mg/g; and in unimplanted valves = 1.2 $\pm$ 0.2 mg/g tissue dry weight. All of the above findings are similar to those in BPV removed from humans. Thus, implanting BPV in sheep provides an excellent means for investigating the preparation and design of the BPV.

Proposed course: Further studies of this project include a comparison of the pathologic alterations that occur in the mitral and tricuspid position, a comparison of the pathologic alterations that occur in bioprosthetic tissues implanted in the subcutaneous and intracardiac position, a study of the morphology of the infection of bioprosthetic valves, and to investigate the second generation valves now developed to retard or eliminate the degenerative processes.

Presentations:

Barnhart, G.R.: An experimental investigation of failure of Hancock and Ionescu-Shiley bioprostheses. 54th Scientific Sessions, American Heart Association, Dallas, Texas November 1981.

Barnhart, G.R.: Intracuspal hematomas in porcine bioprosthetic valves: Pathological observations and clinical implications. 54th Scientific Sessions, Amer. Heart Association, Dallas, Texas November 1981

Jones, M. Experimental evaluation of bioprosthetic valves implanted in sheep. International Symposium on Cardiac Bioprostheses. Rome, Italy, May 1982.

Publications:

Barnhart, G.R., Jones, M., Ishihara, T., Rose, D.M., Chavez, A.M. and Ferrans, V.J.: Degeneration and calcification of bioprosthetic cardiac valves. Animal model: Bioprosthetic tricuspid valve implantation in sheep. Am. J. Pathol 106:136-139, 1982.

Ishihara, T., Ferrans, V.J., Barnhart, G.R., Jones, M., McIntosh, C.L., and Roberts, W.C.: Intracuspal hematomas in implanted porcine valvular bioprostheses: Clinical and experimental studies. J. Thorac Cardiovasc Surg 83:399-407, 1982.

Barnhart, G.R., Jones, M., Ishihara, T., Chavez, A.M., Rose, D.M., and Ferrans, V.J.: Bioprosthetic valvular failure: Clinical and pathological observations in an experimental animal model. J. Thorac Cardiovasc Surg 83:618-631, 1982.

Barnhart, G. R., Ishihara, T., Ferrans, V.J., Jones, M., McIntosh, C.L., and Roberts, W.C.: Intracuspal hematomas in porcine bioprosthetic valves: Pathological observations and clinical implications. Circulation - IN PRESS

Barnhart, G.R., Jones, M., Ishihara, T., Chavez, A.M., Jett, G.K., Rose, D.M., and Ferrans, V.J.: An experimental investigation of the failure of porcine aortic and bovine pericardial bioprosthetic valves. Circulation - IN PRESS

Jones, M., Barnhart, G.R., Chavez, A.M., Jett, G.K, Rose, D.M., Ishihara, T., and Ferrans, V.J.: Experimental evaluation of bioprosthetic valves implanted in sheep. Proceedings of the International Symposium on Cardiac Bioprostheses. (Cohn, L.M., Ed.): Churchill Livingston. IN PRESS

Ferrans, V.J., Ishihara, T., Jones, M., Barnhart, G.R., Kravitz, A.B., Boyce, S.W., and Roberts, W.C.: Pathogenesis and stages of bioprosthetic infection. Proceedings of the International Symposium on Cardiac Bioprostheses. (Cohn, L.M. Ed.) Churchill Livingston, IN PRESS

Ishihara T, Ferrans, V.J., Boyce, S.W., Jones,M., and Roberts, W.C.: Structure and pathogensis of cuspal tears and perforations in porcine bioprosthetic cardiac valves implanted in patients. Proceedings of the International Symposium on Cardiac Bioprostheses. (Cohn, L.M., Ed.) Churchill Livingston, IN PRESS.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02716-02 SU

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Late results after operations for left ventricular outflow tract obstruction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Michael Jones, M.D., Senior Surgeon, Surgery Branch, NHLBI
Andrew G. Morrow, M.D., Chief, Surgery Branch, NHLBI

Other: Glenn R. Barnhart, M.D., Clinical Associate, Surgery Branch, NHLBI
Thomas J. VonRueden, M.D., Clinical Associate, Surgery Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Surgery Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung & Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1-3/4	1-3/4	

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have operated upon more than 130 children and young adults for congenital forms of left ventricular outflow tract obstruction. The present study involves the late results of these operations performed at our institution and a review of the published results from other institutions. Patients included in the study were aged 1-18 years old at operation who had been followed five or more years postoperatively. Forms of left ventricular outflow tract obstruction included valvular aortic stenosis, discrete and diffuse fibrous subaortic stenosis, and muscular subaortic stenosis. Operative mortalities were low (0-6%). However, the operations appear to be palliative ones because only approximately 50% of patients have satisfactory late results.

979

PHS-6040
(Rev. 2-81)

Description: We evaluated the late results of operations for the relief of left ventricular outflow tract obstruction (LVOTO) in young patients, 1-18 years old, from our institution who have been followed for at least five years and from studies in the recent literature which had average follow up durations of five or more years. Operative mortalities for our series and those series we reviewed were low: 1.9% of 522 patients with valvular aortic stenosis (VAS), 6.0% of 222 patients with fixed subvalvular aortic stenosis (SAS) and 5.5% of 18 patients with hypertrophic subaortic stenosis (HSS). From our series, gradients early postoperatively were decreased to less than 40 mm Hg in 88% (30/34) with VAS, in 68% (15/22) with SAS and in 88% (8/9) with HSS. Late survivals for patients in the combined series were: 90% (472/522) for VAS, 86% (190/222) for SAS and 82% (14/17) for HSS, after mean follow up periods of 5-14.4 years. All of our late survivors have had symptomatic improvement; 95% (58/61) are asymptomatic. However, for our patients actuarial analysis predicts that 40 $\pm$ 8% of those with VAS and 44 $\pm$ 10% of those with SAS after ten years will be free from the adverse postoperative events of residual and/or recurrent LVOTO, clinically significant aortic regurgitation, reoperation, endocarditis or late death. Using the same adverse postoperative events to determine satisfactory late results from the combined series, we found that 54% (381/522) of those operated upon for VAS, 54% (120/222) of those operated upon for SAS and 78% (14/18) of those operated upon for HSS had satisfactory late results 5-14 years after operation. Of our patients having unsatisfactory late results, major hemodynamic abnormalities were detected in 55% (23/42) within one year postoperatively. Thus, it appears that operations for most children with LVOTO are palliative ones. These patients should have early postoperative assessments and continuing long-term follow up evaluations during childhood, adolescence and adulthood.

Proposed Course: The patients in this study will continue to be followed and additional patients will be added to the study as they undergo operations. The purpose of the on-going studies is to further delineate the natural history of these operatively treated cardiac abnormalities with the objectives of eliminating or alleviating unsatisfactory late results.

Publications:

Jones, M., Barnhart, G.R., and Morrow, A. G.: Late results after operation to relieve obstruction to left ventricular outflow. In: Symposium on Postoperative Congenital Heart Disease in Adults. (Engle MA, Perloff JK, Eds). York Medical Books, New York - IN PRESS

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 02717-02 SU

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
The influence of Minoxidil on Regional Myocardial Blood Flow and Cardiac Pathology in Beagle Dogs

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: G. Kimble Jett, M.D., Clinical Associate, Surgery Branch, NHLBI

Other: Michael Jones, M.D., Senior Surgeon, Surgery Branch, NHLBI
Victor Ferrans, M.D., Chief, Ultrastructural Section, Pathology, NHLBI
E. Herman, Ph.D., Division of Drug Biology, Food & Drug Administration

COOPERATING UNITS (if any)
Pathology Branch, NHLBI
Division of Drug Biology, Food & Drug Administration, DHHS

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INSTITUTE AND LOCATION
National Heart Lung & Blooc Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The influence of minoxidil on cardiovascular hemodynamics and on regional myocardial blood flow in beagle dogs was investigated. Minoxidil caused increases in mean right atrial and left ventricular end-diastolic pressures. Systemic and pulmonary vascular resistances were reduced; cardiac outputs were increased. Left ventricular stroke work and the tension time index were unchanged by minoxidil administration. Regional myocardial blood flow was measured using radioactive microspheres. Minoxidil increased coronary blood flow to all regions of the heart except to the left ventricular papillary muscles, in which, it was unchanged in comparison to blood flow in untreated animals. Minoxidil increased blood flow to left ventricular subendocardial tissue but significantly less than corresponding areas of subepicardial tissue, reducing the subendocardial/subepicardial blood flow ratio.

981

PHS-6040
(Rev. 2-81)

Description: Ten adult beagle dogs, weighing between 8 and 12 kg, were given two doses of minoxidil (3 mg/kg) 24 hours apart. Shortly thereafter they were anesthetized with alpha chloralose (60 mg/kg, intravenously) and the heart exposed through a left thoracotomy. Aortic, left atrial and left ventricular pressures were recorded. Cardiac output was measured by the thermal dilution technique utilizing a flow directed catheter inserted through an internal jugular vein and positioned in the pulmonary artery. Regional myocardial blood flow was measured using radioactive microspheres (^{46}Sc) 9±1 u in diameter. At the conclusion of the study the heart was arrested with potassium chloride, excised, washed and placed in 10% buffered formalin for three days. The heart was then separated into atria and ventricles. The epicardial fat and epicardial coronary vessels were discarded. The atria were divided into right, left and septal portions. The ventricles were divided into right ventricular outflow and inflow (body) portions, right side of ventricular septum, left side of ventricular septum, anterior and posterior portions of left ventricular free wall, and anterior and posterior papillary muscles of the left ventricle. The ventricular free walls were further subdivided into subendocardial and subepicardial layers of equal thicknesses. Each of the above regions will undergo complete histologic evaluation.

A second group of ten beagle dogs were given a placebo instead of minoxidil. These animals served as controls and were used for comparison with the minoxidil treated group.

Results: Results are expressed as mean values ± standard errors of the means.

Blood Flow (cc/min/gm)

	Ant. Pap. Muscle	Post. Pap. Muscle
Control	1.90 ± .21	1.85 ± .18
Minoxidil	2.32 ± .41	2.21 ± .25

Anterior

	LV Endocardium	LV Epicardium	Endo/Epi
Control	1.41 ± .06	1.46 ± .06	0.91 ± .03
Minoxidil	**2.23 ± .13	**2.91 ± .15	**0.77 ± .04

Posterior

	LV Endocardium	LV Epicardium	Endo/Epi
Control	1.54 ± .08	1.50 ± .08	1.03 ± .02
Minoxidil	**2.72 ± .13	3.48 ± .15	**0.79 ± .06

Hemodynamic Parameters

	HR beats/min	CO cc/min/kg	AO mm Hg	LV mm Hg
Control	*134 ± 5	230 ± 20	125/88 ± 4/3	118/5 ± 5/1
Minoxidil	**152 ± 14	**340 ± 20	**118/60 ± 7/5	**103/14 ± 7/1

	SVR dyne sec cm^{-5}	PVR dyne sec cm^{-5}	LVSW gm m/kg/beat	TTI mm Hg sec/min
Control	3272 ± 287	307 ± 17	2.34 ± 0.2	960 ± 74
Minoxidil	**1376 ± 156	**143 ± 20	1.91 ± 0.13	980 ± 100

** P < 0.05

982

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02719-01 SU

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Computer Simulation of the Cardiovascular System

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Robert D. Moses, Clinical Associate, Surgery Branch, NHLBI

OTHER: Michael Jones, Senior Surgeon, Surgery Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Surgery Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung & Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A computer model of the human cardiovascular system has been developed for the study of pressure-flow-volume dynamics. The principal purpose of the design is to understand the complex interactions between elements of the circulatory system. Reasonable accuracy of performance has been achieved by basing the elements of the model on physiological and anatomical rather than empirical (curve-fitting) properties of the actual system. The principal component is the time-varying elastance model of the heart. Options for varying physiological states, and multiple acquired and congenital abnormalities are incorporated. Graphic output provides an additional understanding of the model's function.

983

PHS-6040
(Rev. 2-81)

Description: A computer model has been developed which is helpful in comprehending the interactions within the cardiovascular system. Initially, an analogous electrical circuit was constructed. This system was converted to the appropriate equations for use by the computer program. The model has as its principal element the time-varying elastance model of the heart which is a representation of cardiac function. It has been demonstrated that the time-varying elastance assumes a state which does not vary with external load changes (preload, after load), but varies sensitively and accurately with intrinsic (contractility). Its simplicity allows it to be readily incorporated into the circulatory model. A lumped element model of the systemic and pulmonary vascular trees has been used which utilizes known values of resistance, capacitance and inertance within the arterial, arteriolar, capillary, venular and venous segments of the circuit. Pressure, instantaneous flow, and individual and regional volumes of the circuit elements can be examined. Altered physiological states can be modeled by changing parameter values. Alterations of heart rate, contractility, diastolic compliance, preload, afterload, total blood volume, vascular dead space, cardiac electrical intervals and valve closure time delay can be studied. The effects of tamponade, heart block, bundle branch block, fibrillation, intrathoracic pressure changes, cardiac aneurysms, AV fistulas, valve stenosis and regurgitation, and cardiovascular shunts can be studied as well. Graphic output via a plotting program and printed output are available.

Results: The model has provided a method of understanding cardiovascular function in a variety of states as described above. Cardiac function, which is the principal emphasis of the model, has been approximated by this computer model.

Proposed Course: Future developments include provisions for more acquired and congenital anomalies, and simplicity of usage.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02720-01 SU

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Temporary relief of pulmonary regurgitation with a balloon "valve"

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Leland G. Siwek, M.D., Clinical Associate, Surgery Branch, NHLBI

OTHERS: Robert E. Applebaum, M.D., Clinical Associate, Surgery Branch, NHLBI
Michael Jones, M.D., Senior Surgeon, Surgery Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Surgery Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung & Blood Institute

TOTAL MANYEARS: 1-1/2	PROFESSIONAL: 1-1/2	OTHER:

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In the course of surgical correction of several forms of congenital heart disease, the pulmonary valve may be rendered regurgitant. Over the long term this is very well tolerated. In the acute postoperative period, however, right ventricular failure is a significant and potentially life threatening complication. While the cause of RV failure may be multifactorial, the extra work produced by the pulmonary regurgitation may worsen the RV failure. This study evaluated in laboratory animals the use of a balloon device to occlude the pulmonary artery during diastole and thus prevent pulmonary regurgitation. The balloon is connected to the control panel for an intra-aortic balloon pump to control the inflation and deflation of the balloon. In 8 sheep the pulmonary valve was completely excised. This resulted in pulmonary regurgitation which was compensated by increased stroke volume and greatly increased RV stroke work. Use of the pulmonary artery balloon, functioning as a "valve", completely prevented regurgitation and reduced RV work.

985

PHS-6040
(Rev. 2-81)

Description: Pulmonary regurgitation was produced in 8 sheep by complete excision of the pulmonary valve. This produced an increased forward stroke volume to compensate for the 40% regurgitant fraction. The pulmonary artery pulse pressure was increased, primarily by a drop in the PA diastolic pressure. The pulmonary flow pattern was monitored via an electromagnetic flow probe on the main PA. The forward and backward (i.e. regurgitant) flow was calculated by planimetry of the area under the flow curve above and below the zero level. The use of a balloon "valve" to occlude the pulmonary artery during diastole resulted in complete elimination of pulmonary regurgitation. The PA pulse pressure was decreased from an average of 22.7 to 11 mm Hg when the "valve" was functioning. Use of the valve decreased the stroke volume from an average of 47 to 26 cc/beat and decreased the stroke work from an average of 11 to 5.25 gm M/beat.

The use of a simple, inflatable balloon to occlude the pulmonary root, controlled by readily available equipment for intra-aortic balloon pumping, provides a method for temporary relief of pulmonary regurgitation. This greatly reduces RV work and hence may be of help to reverse RV failure. With time the RV should recover and then tolerate the regurgitation over a long time. This temporary "valve" device could avoid the need for implantation of permanent prosthetic valves with the known complications they carry in children.

Proposed Course: The study is on-going.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02721-01 SU

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Comparison of dopamine and dobutamine in the early postoperative management of patients undergoing mitral valve replacement.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Leland G. Siwek, M.D., Clinical Associate, Surgery Branch, NHLBI

OTHER: Glenn R. Barnhart, M.D., Clinical Associate, Surgery Branch, NHLBI
Michael Jones, M.D., Senior Surgeon, Surgery Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Surgery Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung & Blood Institute

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1	1	

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Low cardiac output syndrome is a common clinical problem early after mitral valve replacement. In addition, these patients often have increased pulmonary vascular resistance (PVR) which may be exacerbated by commonly used inotropic agents. This study compared the hemodynamic effects of dopamine and dobutamine, over a range of therapeutic doses, in 10 patients within several hours after mitral valve replacement. Both dopamine and dobutamine are effective inotropic agents, producing progressively increasing cardiac output and arterial pressure with increasing drug dosages. Both agents also cause moderate tachycardia and we did not demonstrate any difference in this effect between the two drugs. While dobutamine had no effect on PVR, there was a significant decrease in PVR with dopamine infusion.

987

Description: Ten patients who underwent mitral valve replacement were studied on the first afternoon after operation. While low cardiac output was not a contraindication to study, patients who were dependent on pressors other than dopamine or dobutamine were excluded from study. Hemodynamic monitoring was performed as per the standard routine for these patients. All patients had an arterial catheter for continuous monitoring of arterial pressure and a Swan-Ganz catheter for monitoring of right atrial and pulmonary artery pressures. Cardiac outputs were determined by thermodilution technique via the Swan-Ganz catheter. Left atrial pressure was monitored directly via indwelling left atrial catheters. Systemic vascular and pulmonary vascular resistance were determined by standard formulas.

After a 30 minute control period, drug infusion was begun. The infusion drug was infused at doses of 2, 4, 6, 8, and 10 ug/kg/min, for 5 minutes at each dose. All hemodynamic parameters were measured at the end of each 5 minute interval. There was a second 30 minute control period between drugs, and the order of drug infusion was alternated randomly.

Both dopamine (P) and dobutamine (B) produced significant increases in cardiac index 1.68 $\pm$ 0.26 to 2.44 $\pm$ 0.38 L/min for P, and 1.61 $\pm$ 0.23 to 2.51 $\pm$ 0.38 L/min for B(control vs peak effect). Both drugs produced slight, but insignificant, increase in LA pressure and slight decrease in RA pressure ($p < 0.05$ only at 10 mg/kg/min). Mean arterial pressure was significantly increased by both drugs 84.7 $\pm$ 3.7 to 105.1 $\pm$ 4.9 mm Hg for P, and 85.1 $\pm$ 3.9 to 102.2 $\pm$ 5.5 mm Hg for B (control vs peak effect). Pulmonary artery mean pressure (PAM) was significantly increased by both dopamine and dobutamine, although dobutamine increased PAM at lower dosages. Despite claims that dobutamine is less chronotropic than dopamine, we found no difference between the drugs in this regard. Heart rate increased from 87.6 $\pm$ 9.1 to 119.2 $\pm$ 10.6 beats/min with dopamine and from 90.4 $\pm$ 8.9 to 128.1 $\pm$ 10.9 beats/min with dobutamine (control vs peak effect). Pulmonary vascular resistance (PVR) was not significantly changed by dobutamine (418 $\pm$ 150 control dynes-cm.sec^{-5} vs 395 $\pm$ 97 minimum value). Dopamine, however, produced significant decreases in PVR (408 $\pm$ 91 dynes.cm.sec^{-5} control vs 308 $\pm$ 78 minimum value). Systemic vascular resistance (SVR) was significantly decreased by both drugs (2502 $\pm$ 377 dynes.sec.cm^{-5} to 2131 $\pm$ 285 for dopamine, and 2527 $\pm$ 335 to 2120 $\pm$ 344 for dobutamine). Thus, the only demonstrable hemodynamic differences between P and B in the present study was in their effects upon PVR.

Proposed Course: The study is on-going.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02722-01 SU

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Operations for left ventricular outflow tract obstruction and coronary artery bypass graft or valve replacement

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

none

LAB/BRANCH
Surgery

SECTION

INSTITUTE AND LOCATION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

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XX (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Nine patients have undergone combined operation for left ventriculomyotomy and myectomy and coronary artery bypass grafting. There are 8 men and one woman in the group and their ages at the time of operation ranged from 42 to 64 years. Three had a single bypass graft, 4 had double bypass and 2 had triple grafts (17 grafts total) There was one early postop death from mediastinitis. Four patients have been operated upon since September 1981 and followup is not yet available. Seven patients have undergone combined operation for left ventriculomyotomy and myectomy and valve replacement. Three patients had mitral and four had aortic valves replaced. Two men and one woman had mitral valve replacement (ages 18 to 46 years) and three women and one man had aortic valve replacement (ages 51 to 64 years).

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(Rev. 2-81)

Combined LVM & M and CABG

Three patients had a single coronary artery bypass, four had double bypass and two had triple bypass (17 grafts total). There was one early postop death (less than 30 days) from mediastinitis. Four patients have been operated on since September 1981 and follow up is not yet available. Follow up ranging from 1.5 to 6.5 years is available on four patients with eight grafts (one single, two double, one triple). All four patients had good relief of obstruction and five of the eight grafts were patent at the time of postop catheterization. Two of these patients are alive and NYHA FC II 1.5 and 3 years after operation. One of these patients had undergone SCABG and the graft was occluded at the time of postop cath. He has some residual angina. The other patient underwent TCABG and all grafts were patent. This patient has had no further angina.

The other two patients died 2 and 6.5 years after LVM & M and double coronary artery bypass grafting. In each of these patients one graft was patent at the time of postop study. Both patients died suddenly. The patient surviving 2 years was hospitalized at the time of death for evaluation of metastatic lung cancer and was found dead in his room. No necropsy was performed but was known to have had CHF symptoms and arrhythmias prior to his final illness. The other patient died suddenly at home and had suffered recurrent angina for about 4 years prior to his death. At necropsy both grafts were occluded.

Coronary artery disease after left ventriculomyotomy & myectomy: Two patients who underwent only LVM & M returned late postop for evaluation of angina and were found to have significant coronary artery disease. One of these patients underwent CABG ten years after myotomy & myectomy. He died intra-operatively. The other patient presented 8.5 years post myotomy & myectomy and he had a PTCA performed.

Combined LVM & M and Valve Replacement

Mitral valve: There were two men and one woman in this group and their ages at the time of operation ranged from 18 to 46 years. All three had mitral regurgitation. One patient had ruptured chordae; one had a congenital deformity of the mitral valve; the third patient had a grossly normal appearing valve which, however, was significantly regurgitant and had to be replaced before the patient could be weaned from bypass. There was one perioperative death. A second patient died 4 years postop in congestive failure. A catheterization obtained shortly before her death was consistent with congestive cardiomyopathy. The third patient is alive and NYHA FC II seven years postop.

Aortic valve: There are 3 women and one man in this group and their ages at the time of operation ranged from 51 to 64 years. All four patients had aortic regurgitation on preop catheterization and two of the four had a history of endocarditis. At operation one patient with previously treated endocarditis was found to have a perforation of one of the aortic valve leaflets. The second patient with a history of endocarditis had a grossly retracted valve. The third patient had a bicuspid calcified valve and the fourth patient was found to have AV leaflets that were thickened, shortened, and contained some calcium. All four patients in this group survived operation. Three of the four underwent postop cath and were found to have good relief of their outflow obstruction.

ANNUAL REPORT OF THE
LABORATORY OF TECHNICAL DEVELOPMENT
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
OCTOBER 1, 1981 TO SEPTEMBER 30, 1982

Microchemical Instrumentation

This year's efforts were devoted to two separate activities. The first involved finishing the testing and evaluation of the flow-through microfluorimeter. The second concerned the development of a new fiber-optic PCO_2 sensor useful for tissue as well as blood PCO_2 measurements. Both projects fall into the general area of micro chemical sensors. Last year the work on the flow-through fluorimeter had progressed to the point that we had shown that a fluorimeter could be made with a sensitive volume of 250 nl and the ability to detect femtomole amounts of fluorescent substances. This instrument uses a glass fiber optic to carry ultraviolet light from a mercury lamp to the quartz capillary cuvette. By using the fact that the light leaving the fiber optical fiber is well collimated, we could keep the fluorescence excitation energy concentrated in the sample and reduce the possible scattered light which could obscure the fluorescence signal. The current year's work was devoted mainly to the elucidation of the details needed to make a working assay system for picomole amounts of ammonia. By appropriate selection of buffer, enzyme concentration, substrate and cofactor concentrations, we were able to obtain consistent sensitivities below one picomole. The staff of the LKEM is now using the instrument for the study of the production and transport properties of rat kidney tubule segments for ammonium ions. There are no immediate plans for further development of this project but there is some commercial interest in making the microfluorimeter.

The second major activity, the fiber optic pCO_2 sensor, was developed during a six month's sabbatical at John Severinghaus's Anesthesia Research Group, Cardiovascular Research Institute, Univeristy of California, San Francisco. The fiber-optic approach offered a way to solve the problem of estimating the tissue pCO_2 at the medullary respiratory control center; the group had been struggling to develop an instrument based on the conventional pH electrode approach for some time. The sensor consists of a small volume of bicarbonate buffer with phenol red dye contained in a piece of CO_2 permeable silicone rubber tubing. The total volume is less than one microliter. One end of the tubing is plugged with white silicone rubber cement, which acts as a reflector for light which is introduced into the other end of the tube by a 125 micrometer dia. plastic fiber optic. A second similar fiber collects light from the reflective surface and carries it back to a photosensor. The transmitted signal at 570 nm, near the absorption maximum of the base form of phenol red, is directly proportional to the pCO_2 over the range from 20 to 100 mm Hg. The overall length of the probe is about 60 cm from the tip to the bifurcation where the two fibers diverge to go to the light source and detector. The fiber optic pCO_2 sensor offers a simple, linear, and small sensor for measurement of local tissue pCO_2. The 95% response time is a minute, and it has good long-term stability, drifting less than 2 mm Hg over a 10 hour experiment.

It seems possible to reduce the size of the sensitive region about ten fold by using a single smaller fiber and a micropipetlike buffer container. Both the fiber-optic pCO_2 sensor and the microfluorimeter represent significant advances in areas of instrumentation involving chemistry and optics in very small spaces.

Cell Measurements Systems

Work on methods and apparatus for the study of kidney and toad bladder epithelial cells grown as sheets on porous membranes continues to yield important results. The central element is the porous bottomed culture dish (PBCD) in which the membrane is cemented to polycarbonate ring which provides electrical and chemical isolation between the solutions contacting each side of the membrane. With these PBCDs new knowledge of development of epithelia is being obtained.

PBCDs with very permeable bottoms allow exchange of nutrients and waste from both sides and results in a response to vasopressin by A6 cells (from Xenopus laevis kidney) which does not occur otherwise. However, the cells continue to grow to the limits of this nutrient supply and eventually become very sensitive to stirring and medium replacement. This growth can be controlled by using medium with no serum added on the apical side (urine side) and the usual medium 10% fetal bovine serum on the basal side (blood side). The ability to manipulate the 2 sides of the preparations separately offers great promise for the study of the development of epithelia. This is particularly true when one uses transparent support membranes such as the collagen membranes which we have developed. One can watch the A6 cells spread across the membrane and become confluent. The PD does not develop at this time. The cells go through many gyrations (moving and changing shape) before the PD develops a couple of days later. These gyrations are much simplified and shortened when medium with no serum is used on the apical side. The collagen membrane PBCDs are proving useful for developing primary cultures from several sources (rabbit kidney, rat kidney and toad bladder).

Due to the important role which calcium ions appear to play in many cells including epithelial cells, a project has been started with the objective of improving the specificity of the Ca^{++} ion selective electrode. The calcium activity inside most cells is very small: less than 10^{-6} molal. In fact, most experimental values are probably too high. Values obtained using dyes being high because these dyes have affinities for Ca^{++} comparable to some of the things inside the cells which bind the Ca^{++}. The ion specific electrode reads high due to interferences of other ions such as K^+, Na^+, and Mg^{++}. These interfences are particularly bad for the small microelectrodes needed to successfully enter epithelial cells (<0.1 micron tip diameter). The changes being explored to obtain improved specificity are: (1) Uses of glasses without diffusable ion (fused quartz and Corning 1723). (2) Silanization of the glass with monochloro-silanes with long hydrocarbon chains with careful exclusion of water vapor. (3) Explore attachment of the Ca^{++} exchange molecules directly to the glass.

Separation Science Instrumentation

Development and application of countercurrent chromatography has been continued. A newly developed countercurrent chromatographic scheme performs efficient preparative-scale separations in a short period of time. The scheme uses a particular type of the coiled column called "multi-layer coil" which consists of multiple layers of the coil as seen in a reel. The column is subjected to a synchronous planetary motion to yield an extremely high retention of the stationary phase against the fast flow of the mobile phase. Consequently, the efficient separation is completed in a few hours instead of overnight to days which would be required by other CCC methods. The capability of the new CCC method has been demonstrated in separations of various biological samples such as DNP amino acids, natural and synthetic peptides, purines and pyrimidines, indole plant hormones and abscisic acid, etc. For separation of cells and macromolecules, a new non-synchronous flow-through coil planet centrifuge has been constructed. The design of the apparatus eliminates the use of spider gear sets which produced a considerable noise and vibration in the previous model to limit the practical use. The new centrifuge can be smoothly run up to 1000 rpm combined with a slow coil rotation which is freely adjustable anywhere between 0 and 50 rpm in either direction. Capability of the apparatus has been demonstrated in partition of macromolecules and cells on polymer phase systems and elutriation of mammalian cells with physiological solutions.

Biophysical Instrumentation

The Section on Biophysical Instrumentation has been engaged for several years in developing a series of microcalorimeters aimed at providing the biomedical scientist with new tools for exploring biochemical reactions in solutions and cells where no other method of observing the reaction is available other than the evolution or absorption of heat. In cooperation with the Biomedical Engineering and Instrumentation Branch, DRS, a new microcalorimeter has been developed especially for thermal kinetics. In past instruments various artifacts limited the ultimate sensitivity that could be reached. In this instrument, a reaction heat of 5×10^{3} cal/mole would allow the investigator to determine this heat with only 2×10^{-8} moles of reactants. Thermal kinetics could be done on considerably less protein. Work on several Ca dependent reactions have been started with investigators in NHLBI and NIA. In order to better understand the reactions under investigation a differential pH thermal titration apparatus has been constructed and used to study the reaction of Ca with phytic acid. Work on calmodulin and EGTA, a calcium buffer used for studies of muscle physiology because of its much greater affinity for Ca over Mg, is also under way with this instrument. Both of these instruments are under microprocessor control.

At the request of several investigators we have been working on a small simple, flow apparatus that will allow two solutons to be mixed together quickly and then sprayed at a cold plate, thus quickly quenching the chemical reaction being studied. We have now constructed such a device using our highly efficient ball mixers which are expecially designed to handle cells, membranes, sacroplasmic reticulum, etc., even at high viscosities, without destruction at velocities to 10 meters/second. This unit is a 3 syringe drive sytem which allows the ratioing of syringe A to

syringe B to be varied from 1:1, 1:2, 1:5, 1:10, and 1:20. Two mixers are used. Test reactions are presently being run with time resolution of freezing in the 5 to 10 millisecond range. A portable version of our NBS traceable Temperature Standard has been constructed with the aid of BEIB-DRS and calibrated at NBS. One of the units will form the temperature reference standard being set-up at the Center for Infectious Diseases, CDC at the request of the World Health Organization. This unit provides the requisite ± .01°C absolute temperature reference from 0°C to 60°C.

Luminescence Instrumentation

Several advances in methodology have been made. 1. Our dye-liposome assay for insect venom peptides which cause membrane lysis has been extended to certain antibiotics such as gramicidin and polymyxin. Ion channel-formers cause dye leakage. 2. The so-called "refractive index correction factor" for fluorometry has been analyzed and experimentally tested. No evidence for any need for such a correction was found for a standard geometry spectrofluorometer. 3. The steady-state fluorescence depolarization method yields rotational relaxation times by measuring the amount of depolarization occurring in the interval between absorption and emission of polarized light. The fluorescence lifetime must be neither too short nor too long, but an "optimum" lifetime figure had never been calculated. Taking into consideration photometric error and limiting polarization, we calculated the amount of error in relaxation time as a function of the lifetime, thereby calculating the optimum lifetime and the way the precision of the assay varied with lifetime. Surprisingly, it was shown that lifetime which were only 1/25 as long as the rotational relaxation time were suitable, if not optimal, for such depolarization experiments. This was confirmed with examples, namely, proteins of various sizes, using the intrinsic ultraviolet fluorescence from tryptophanyl residues.

Fluorescence has been applied to certain problems of interest in biophysics. 1. Proteins are now recognized to have a dynamic structure which flutuates constantly, in contrast to the essentially static picture given by the X-ray crystallographers. To measure segmental motions, we have used fluorescence polarization with dye conjugates of proteins. Polarization has been measured isothermally, as well as in heated solutions. The results, coupled with fluorescence lifetimes data, show that the "global" rotation of the whole protein is given by the isothermal experiments, while thermally activated rotations are clearly shown in the heating experiments. The effective size of the rotating subunit decreases with temperature, indicating increased degrees of freedom. 2. Serum albumin is perhaps the most abundant protein in the body. Its interaction with drugs such as aspirin is of medical importance. Aspirin is acetylsalicylic acid, and like other esters, has been shown by others to acetylate serum albumin. We have found that certain nonfluorescent, but fluorogenic, acetates such as 4-methyl-umbelliferone actetate also acetylate albumin. The rates of reaction have been found to be unusally rapid; the rates were measured by manual and stopped-flow methods. These fluorogenic acetates serve as models for acetylating-type drugs, and serve also as a probe of a specific site in albumin to enhance our knowledge of the structure of this protein. 3. In collaboration with Dr. Loewenthal, on sabbatical from Jerusalem, we have

measured the phosphorescence and delayed fluorescence of biphenyl and naphthalene in frozen cyclohexane. The purpose is to measure the amount of diffusion allowed in this frozen but plastic matrix, since delayed fluorescence arises from triplet-triplet annihilation. Armed with knowledge of matrix characteristics, Dr. Loewenthal plans to extend the work with ionizing radiation in such a way as to be able to calculate the density of excited molecules in the matrix, as a function of radiation doses. 4. In collaboration with Dr. Hantgan and Dr. Liesegang we have measured the fluorescence decay and anisotropy decay of a derivative of fibrinogen. This molecule of 340,000 has been shown to have internal segmental flexibility which is necessary for its function in coagulation. The results quantitate the amount of segmental motion and are inconsistent with any model of the protein as a large rigid structure.

Picosecond Spectroscopy

The work is divided into 3 areas: 1. Vidicon methodology. 2. Picosecond laser photolyis. 3. Fluorescence decay time technology.

1. A thorough investigation of the vidicon as a quantitative spectroscopic detector under both continuous and pulsed illumination has been completed. As a result of this study techniques have been developed which now allows the use of the full dynamic range of vidicons. Results of this work are being used by several NIH investigators as well as Princeton Applied Research Corporation requesting to use our findings in a series of application notes and incorporating several of our techniques into their OMA2 system.

2. The development of several flash photolysis instruments for initiating and detecting the primary photoprocesses occurring in biological systems has been completed. The instruments provide short pulse excitation (< 20 picoseconds) tunable excitation from the deep UV to IR with energies up to several millijoules and extensive signal averaging capabilities. The first is a high energy (2mj) low repetition rate (0-6ppm) instrument. Through the development of a novel double-beam configuration utilizing fiber optics a $\pm$ 0.02 Δ OD sensitivity has been achieved. To further improve the signal-to-noise ratio and minimize intensity fluctuations in the excitation source the laser has been successfully operated at a repetition rate of six pulses per minute (this being a factor of ten faster than other existing systems) thus allowing signal averaging of the spectra. Optical density changes of $\pm$ 0.01 or better were routinely obtained as well as a significant reduction in the time required to obtain a complete photolysis spectrum. The second photolysis system is low energy (100 microjoules) high repetition rate (0-100 pps) instrument. In preliminary experiments this system is proving highly reliable and sensitive thereby making it a viable research tool for the photobiochemist. For example, in photolysis experiments changes in optical density of 10^{-3} are easily detected with a minimum of signal averaging (<5 minutes). This is both an improvement in the signal-to-noise ratio as well as a significant reduction (one to two orders of magnitude) in data acquisition time. Maximal sensitivity in ΔOD through full overlap of the excitation and probe beams is now possible due to the nearly "CW" characteristics of the system. Finally, the use of moderate

excitation energies <1MW/cm^2 reduces the chances for photolysis artifacts common to most picosecond systems. The unique capabilities of these spectrometers will be applied to the study of photobiology with emphasis on the photosensitization of proteins and cells. This will include the photodynamic actions of hematoporphyrin and hematoporphyrin derivative.

3. Most intrinsic fluorescence in proteins occurs in the 0.1 to 10 nanosecond time regime. Present-day fluorescent lifetime instruments require deconvolution of the 2-3 nanosecond lamp pulse, and so add considerable uncertainty to the accuracy of the lifetimes. A fluorescent lifetime system with a 0.2 nanosecond response time has been designed and partially constructed to permit direct measurements of intrinsic and/or extrinsic lifetimes and depolarizations down to 0.4 nanoseconds without deconvolution. This instrument will be applied to the study of motions in proteins and membranes including hemoglobin.

Cardiac and Respiratory Assist Systems

The Section on Pulmonary and Cardiac Assist Devices has continued to explore the cause of acute lung failure in newborns, children, and adults, and in methods to treat this patient population with extracorporeal membrane artificial lung perfusion system. Laboratory studies were carried out in a fetal lamb population (128-130 d gestation) at high risk of developing respiratory failure. Animals treated by state of the art pulmonary care rapidly entered respiratory failure, with pH less than 7.050, $PaCO_2$ over 120 torr, and with PaO_2 less than 15 torr. One group of these animals were continued on mechanical ventilation, and all were dead within a few hours; the remainder of these animals were placed on continuous positive airway pressure (CPAP) at 15 cm H_2O and on apneic oxygenation, and were connected to an extracorporeal perfusion system. Within 5 minutes of beginning bypass the blood gases had markedly improved, and the blood gases became normal within one hour of beginning bypass. There was a progressive rise in total static lung compliance and a clearing of X-ray films. Following some 4-6 hours on bypass, the animals were then placed on mechanical ventilation with continuing improvement in compliance, and lung function. 24 hours after beginning mechanical ventilation, the animals were on room air ventilation. Hence, animals that undoubtedly would have gone on to die, showed rapid improvement in lung function while on our treatment, with full restoration in lung function. Our findings suggest that continuing high distending pressure on the lungs gradually restored normal functional residual capacity and lung compliance, and yet with no measureable change in surfactant sytem. Lungs so treated over a period of hours had thus acquired the ability for normal pulmonary ventilation, and without developing hyaline membrane disease. These findings demonstrate the great capability of the membrane artificial lung perfusions system in reversing a potentially lethal process to one of full recovery.

We have further improved on the extracorporeal perfusion system by making use of a single catheter cannulation method. In this method, blood is intermittently drained and pumped through the membrane artificial lung, and is then returned to the patient through the same venous catheter. Only

one catheter need be placed (and only one vein need be cannulated). This technique substantially simplifies bypass technique, it greatly reduces blood loss, and it very likely represents a major contribution to extracorporeal technology with membrane lung bypass.

Clinical studies in collaboration with Dr. Gattinoni in Milan, Italy, have shown great success treating patients with severe adult respiratory distress syndrome (ARDS) with CPAP and with extracorporeal membrane lung bypass. During the past 2 years, a total of 34 patients entered the study, all of whom met extracorporeal membrane oxygenation (ECMO) entry criteria and with a presumed mortality of 90%. Of those patients treated by applying concepts originating from this laboratory, 73% were long term survivors. These results represent a vast improvement in overall survival and are likely to be improved even further. It is worth noting that in the ECMO ancillary study involving patients who required mechanical ventilation and while on 50% oxygen (although not meeting ECMO entry criteria), but 33% of those patients were still alive one month later. Very likely, the ML respiratory assist when applied to this patient population could have a substantial effect on the ultimate outcome, and survival.

In sheep maintained on cardiopulmonary bypass we investigated the effect of ventilating the lungs with room air, alone or with 1% or 5% CO_2. Those on room air alone showed reduced lung compliance and reduced functional residual capacity within 4 hours while those on CO_2 remained normal during 24 hours of total cardiopulmonary bypass. In the animals ventilated on room air the capillary blood became severely alkalotic (over pH 7.8)and the lungs showed atelectasis and hemmorrhage. As the surviving animals remained normal we infer that the ventilation of hypo perfused lung with air was responsible for the early demise of these animals.

Our findings may be applicable to the reduction of morbidity induced by prolonged total cardiopulmonary bypass.

Clinical Devices

The laser doppler blood flow monitor work and the NMR flow projects are being discontinued. The laser capillary flow monitor is now a NIH licensed commercially available instrument and the NMR flow project is now a government supported project of the Medical College of Wisconsin.

Polyurethane extention topocatheters on 7 french angiocatheters have been tested in animals with excellent results. Twelve centimeters long and one millimeter tip can be made but material defects make production tedious and umpredictable. In a search for more suitable materials reconstituted collagen has performed remarkably in early tests. Non-antigenic reconstituted collagen is especially attractive as the medical use of this material has already been accepted for some applications.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01404-14 LTD

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Membrane Lung Systems for Long Term Respiratory Support

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS: 4	PROFESSIONAL: 3	OTHER: 1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have explored the use of an extracorporeal perfusion system that includes a membrane lung, to the treatment of hyaline membrane disase, adult respiratory distress syndrome, and meconium aspiration syndrome. Lungs were managed by apneic oxygenation while on continuous positive pressure ventilation, and while metabolically produced CO_2 was continuously removed by an extracorporeal membrane lung. Lungs so treated rapidly improved, resulting in a cure and long term survival. The perfusion sytem has been simplified, and now the perfusion can be performed though a single vein cannulation technique.

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PHS-6040
(Rev. 2-81)

Objectives:

1. To develop a perfusion system for long term pulmonary and cardiopulmonary assist.

At present, the majority of open heart procedures are still performed using the bubble oxygenator as the artificial lung. However, for procedures longer than 6 hours the continued use of the bubble oxygenator brings about cumulative deleterious effects that seriously affect the ultimate survival of the patient. No such limitations are found when using the membrane oxygenator (membrane artificial lung) in open heart surgery. Perfusions based on membrane artificial lungs have been successfully performed lasting days or weeks. It is the aim of the program to devise and to improve on a perfusion system for long term applications based on the membrane artificial lung system.

2. To develop a perfusion system for long term extracorporeal removal of CO_2 for the control of breathing and the treatment of respiratory failure.

The concept underlying this project is based on the observation that breathing is tailored not so much for oxygen uptake by the lung, but to CO_2 removal. While it remains true that any severe hypoxia represents a life threatening insult, the mechanics of breathing remains intimately tied to CO_2 elimination by the lungs, and hence the tidal volume and respiratory rates; and in patients on mechanical ventilator, to tidal volume, respiratory rates, and the airway pressures. In lungs severely impaired, the need for high airway pressures to obtain adequate alveolar ventilation can lead to barotrauma, hepatic and renal impairment, and the likely worsening of the clinical picture. It is therefore highly desirable to design a safe system whereby most if not all metabolically produced CO_2 could be removed through other means than the natural lungs, i.e. through artificial means.

The extracorporeal perfusion system can be designed to meet most or all of the needs to remove metabolically produced CO_2 at an extracorporeal blood flow rate but 20% or less of that needed to attain comparable transport of oxygen. Moreover, such a procedure can be performed in a vein to vein perfusion sytem, or even using a single vein cannulation technique.

It is the aim of this program to search for alternate means to restore health to high risk patient groups with severe lung impairment of whatever cause, in newborns, adult, or in children. Such a system based on an extracorporeal membrane lung can be suitable not as a last resort treatment, but may become applicable to preventively treat patients at high risk of progressing to frank respiratory failure.

In newborns, pulmonary disease is the greatest source of morbidity and mortality. The greatest cause of pulmonary dysfunction in newborns is

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immaturity of the lungs, leading to respiratory failure. Other factors involving newborns, such as meconium aspiration, are less frequent, but more lethal.

The respiratory failure in chronic lung disease represents an entirely different underlying cause, and it may be feasible to resort to an approach not unlike what is practiced in chronic renal failure, treated with hemodialysis. Such an approach may help restore blood gases to normal, help alleviate circulatory failure, leading to improvement or restoration in quality of life.

3. Acute respiratory failure: adult respiratory distress syndrome (ARDS), and hyaline membrane disease (HMD).

The poor outlook of patients with ARDS has been well documented through the recent NHLBI sponsored ECMO (Extracorporeal Membrane Oxygenation) study. Briefly, 90% of patients meeting ECMO entry criteria died in the control group, with no improvement in overall results when treatment was augmented through the use of an extracorporeal membrane lung (ML). Even more important is the long term outcome of patients deemed not sick enough to meet ECMO entry criteria, and who in fact never met the entry criteria: 67% of patients died by the end of one month due to a variety of combined organ system failures. Thus while respiratory failure may be the first precipitating cause of the patients' illness, the evoluton of the disease process lead to multiorgan system failure, culminating in death.

We believe that in the majority of cases the evolution of pulmonary disease can lead to full recovery of health, even in those patients in whom the likelihood of survival is but 10% or less. However, clinical approach to treating acute lung failure must be drastically revised. Cardinal among our proposed approach is to discontinue the use of a mechanical ventilator and thus to remove the patient from continued onslaught of high pressure pulmonary ventilation with its many well known effects on organ system function. This can now effectively and safely be performed thorugh the use of an extracorporeal membrane lung perfusion system.

Similarly, in the management of acute respiratory failure in the newborn (HMD) it is important to recognize that lungs of many "immature" newborns now developing HMD have a fully developed pulmonary vascularity and are thus capable of pulmonary oxygen transport. However, these same lungs may not and are not yet fully developed to allow pulmonary ventilation to effect CO_2 removal. The use of an extracorporeal ML perfusion system (the artificial lung) provides for precisely the right means to successsfully remove CO_2 to maintain $PaCO_2$ normal, without requiring respiratory activity. The use of the ML perfusion system thus allows us to buy the time for the lungs to mature and to heal, while maintaining normal arterial blood gases, and without continuous insult on the lungs by mechanical ventilators.

Methods Employed and Major Findings:

1. Within limits, most membrane based artificial lung perfusion systems could be successfully employed for extracorporeal CO_2 removal, and hence respiratory assist. The blood flow required to effect CO_2 removal is small, hence several means have become attractive. In a double lumen venous cannulation the deep femoral vein is cannulated and a thin walled double lumen catheter is inserted allowing for continuous drainage of venous blood, an the continuous return of blood coming out of the membrane artificial lung. Blood flows up to 3 liters per minute have been obtained through this single vein cannulation technique, well beyond the 1-1 1/2 liters per minute flow required in this procedure. The single vein cannulation technique has the advantage of being more rapid to institute perfusion, and in reducing surgery and hence reducing potential bleeding from the wound.

Such cannulation using the double lumen catheter is not possible in newborns with their small veins. Instead, we have now developed and tested a single catheter perfusion system where blood is intermittently drained, and is returned, through the same venous catheter. Our extensive experience makes us believe that this is the appropriate method for use in newborns, and very likely so also in adults. In the latter patient group, this opens the possiblity of cannulating a single saphenous vein and thus requiring little dissection to effect venous cannulation. Such a technique (although very simple) may well represent a major advance to prolonged safe extracorporeal bypass for respiratory assist in adults, and the neonate.

2a. Fetal Work

The animal model used extensively in this laboratory is the fetal lamb of a gestational age between 128-130 days. This population of lambs, using conventional treatment as practiced in clinical medicine, has a mortality of near 100%.

In our previous studies we have shown in fetal lambs still connected to the umbilical circulation and the ewe, that at a CPAP (continuous positive airway pressure) of 15 cm H_2O and with hourly insufflation to a pressure of 35 cm H_2O, the total static lung compliance (TSLC) progressively rose. Once TSLC exceeded 0.6 ml $(cm\ H_2O)^{-1}kg^{-1}$, we felt that lambs were then ready to be delivered and to be placed on mechanical ventilation (MV). All animals survived 24 hours of MV while on room air, in excellent health, and without developing HMD.

In the present study, we used fetal lambs of the same age group and placed them immediately on a mechanical ventilator, providing state of the art pulmonary care. Within 10-20 minutes of such treatment, the lambs developed severe HMD with pH less than 7.05, $PaCO_2$ over 120 torr, PaO_2 10-15 torr. In a control group of animals, treatment was continued by MV, and all animals died within a mean of 2 hours of severe respiratory failure. However, when a similar group of lambs with similar respiratory failure was then placed on an extracorporeal ML bypass system and on CPAP of 15 cm H_2O, there was a rapid improvement in arterial blood gases and a

progressive rise in TSLC. Following some 8 hours of treatment the TSLC had markedly improved and extracorporeal bypass was discontinued, with survival of the animals in good health and on room air.

In another group of fetal lambs the initial ventilation while on MV immediately after delivery was accomplished with 3% CO_2 in 80% oxygen. While respiratory failure following initial MV developed of the same order of severity as when ventilating with 80% oxygen alone, those animals recovered TSLC much more rapidly than when ventilated with 80% oxygen. These findings are of great significance, and may point the way toward preventive treatment in newborns at high risk of developing HMD. For instance, if those newborns at high risk of developing HMD were in an environment containing supplemental CO_2, it is possible that incidence of HMD could be markedly reduced, or even eliminated. The mechanism for the beneficial effect of CO_2 in preventing HMD was suggested from studies on total cardiopulmonary bypass (see below). The fetal pulmonary circulation in utero differs from adult type of pulmonary circulation. After delivery, the transition from fetal to adult circulation can remain incomplete, resulting in areas of severe pulmonary hypoperfusion. We believe that in lungs not perfused (but ventilated), a severe regional capillary and tissue alkalosis invariably results, leading to pulmonary cellular injury, pulmonary atelectasis, and HMD. This scenario represents a new and original concept in the cause, and treatment of HMD.

2b. Meconium aspiration syndrome (MAS).

MAS is a disease of the near term or term newborn with intrauterine fetal distress. Unlike HMD which strikes mostly preterm newborns, MAS affects mostly term newborns. Current state of the art treatment frequently fails.

MAS is named after the color of the green meconium that stains the newborn. Yet one must recall that meconium is invariably aspirated together with amniotic fluid, never without. Interestingly enough, amniotic fluid has never been implicated in causing HMD, the latter term being reserved to premature and immature lungs not capable of adequate pulmonary ventilation.

We have explored the effect of lung lavage with autologous amniotic fluid in fetal lambs. In fetal lambs that would naturally survive in our hands with usual pulmonary care, we found severe RDS developing in 4 out of four fetal lambs so treated, with 3 lambs dying of progressive respiratory failure within four hours. Thus the lavage of these lungs with amniotic fluid reproduced a picture indistinguishable from HMD in this population group of fetal lambs. Studies are now in progress to treat those very same fetal lambs with respiratory failure with the extracorporeal ML perfusion system.

We believe intrauterine amniotic fluid aspiration on a massive scale may be the as yet unrecognized factor that in a very young fetal population can lead to HMD shortly after delivery. Amniotic fluid aspiration on a smaller scale can thus lead to a varied picture ranging from no disease, to various grades of severity of HMD. The finding of meconium in the older near term newborn population my provide the added insult to render those patients, at

some risk already to become high risk conadidates for respiratory failure, although now called MAS.

2c. Total cardiopulmonary bypass (TCPBP) for cardiopulmonary assist.

These studies are designed to explore the limits of the possible for prolonged extracorporeal bypass in cardiopulmonary assist, as during open heart surgery, or for prolonged total cardiopulmonary assist with severe right and/or left ventricular failure.

It is widely accepted that duration of CPBP is associated with increased morbidity and morality, in part related to wide use of bubble oxygenators, but in part related to lung dysfunction.

We provided total CPBP in anesthetized, paralyzed sheep using a membrane lung perfusion system for 24 hours. The lungs were either ventilated with room air, or room air enriched with 1 or 5% CO_2. A Swan Ganz flow directed catheter was placed in the wedge position. In the group ventilated with room air, the PCO_2 of retrograde blood from the Swan Ganz catheter immediately approached zero, with pH over 7.8; in the group ventilated with 1% CO_2, the corresponding PCO_2 was 10 torr and pH 7.70; in the group ventilated with 5% CO_2 the PCO_2 was 35 torr and pH 7.4. The TSLC and functional residual capacity (FRC) were highly abnormal in the group ventilated with room air alone, while both remained normal in the two other groups.

Within two hours the lung function in the group ventilated with room air had deteriorated markedly, the animals became hypotensive, went into shock, and died. At autopsy those lungs were hemorrhagic, and liver like.

The other two grups were successfully managed for 24 hours, and lungs at elective autopsy appeared unremarkable.

These studies point to the ventilating gases used to ventilate the lungs as the single variable resulting in either good lung function, or severe and lethal impairment in lung function with death. It suggests that lung tissue alkalosis of this magnitude is the critical element to the continued integrity of the lungs as a functioning organ. These studies suggest that perceived limits to long term bypass have been in part due to inattention to the management of the lungs in a proper manner. Enhancing the inhaled gases with some added CO_2 may greatly expand the survivability of patients on prolonged CPBP

2d. Treatment of ARDS

These clinical studies were carried out in collaboration with the Institute of Anesthesia and Intensive Care in Milan, Italy.

Up to date, 34 patients with ARDS, and all meeting ECMO entry criteria, have been treated with Low Frequency Positive Pressure Ventilation and Extracoporeal CO_2 Removal (LFPPV-ECCO$_2$R), the technique that was first applied to animal research in this laboratory. Patients were first

ventilated with inverse ratio ventilation (IRV) during which time they were assessed for TSLC. Those with TSLC above 30 ml $(cm\ H_2O)^{-1}$ were successfully placed on continuous positive airway pressure (CPAP), and all but one out of 14 patients is a long term survivor; 9 patients had a TSLC between 25 and 30 and were on IRV for a few more days, and 5 were eventually weaned to CPAP and survived; the 4 remaining, together with 12 other patients with TSLC below 25 were placed on extracorporeal ML bypass, with 63% surviving. The overall survival among the 34 patients meeting ECMO entry criteria (10% survival with state of the art treatment) was 73%.

These results are greatly better than those of the ECMO study. Our results point out the importance in discontinung MV treatment, when it becomes obvious from TSLC measurements that further MV is futile, and in our opinion, lethal.

It is well worth noting the rapidity of improvement after beginning ML bypass. In most patients the PaO_2 exceeded 100 torr within the first 12-24 hours, and FIO_2 could be reduced to 0.4 - 0.6. There was usually a massive diuresis associated with concurrent improvement in arterial blood gases. The duration of the treatment before discontinuing bypass averaged 6.4 days, and was in part related to the duration of the preceeding intensive ventilator care at the referring institution.

Hence, research in this laboratory has led us to greater understanding of the pathogenesis of ARDS, and has opened to us the doors to better treatment in ARDS, with spectacular improvement in overall survival.

Significance to Biomedical Research and the Program of the Institute:

1. Respiratory failure in any hospital setting is a serious disease. Patients may have contracted respiratory disease outside the hospital, or as part of their hospital course. We believe the understanding of the mechanism of lung injury during MV is important to the treatment of patients if significant improvement in survival is to be achieved. It may no longer be acceptable to apply to diseased lungs a higher and higher airway pressure to effect CO_2 elimination. Rather, a simple perfusion system devised in this laboratory may be all that is needed to wean the patient off MV and to CPAP, with metabolically produced CO_2 removed by an extracorporeal ML. What appears to be needed is not better and improved MV, but an understanding of the many variables that led to respiratory failure once certain underlying conditions are met.

The hazards of tissue injury due to marked capillary alkalosis in states of pulmonary hypoperfusion can readily be avoided through the addition of some CO_2 to the ventilating gases. This simple maneuver may be all that is needed to safely prolong CPBP, to markedly reduce postoperative lung dysfunciton following CPBP, and to reduce incidence of ARDS.

2. We believe newborns at high risk of developing HMD can be managed with less risk and much better prognosis. The entity of Bronchopulmonary Dysplasia (BPD) is the direct result of aggressive pulmonary treatment with high airway pressures and elevated oxygen concentrations. We believe that the assessment of the patient's TSLC to be the single most important test to help decide on the ultimate course of treatment, and whether an extracorporeal membrane lung may be indicated. Laboratory experience suggests that most patients can be weaned off bypass in 24-48 hours.

The prophylactic use of added CO_2 in the inhaled gases in the newborn nursery deserves careful consideration. The sparing effect on lung injury noted in the animal studies suggests that a similar sparing action may be found in those at risk newborns in whom breathing during the first few hours of life often is without any obvious difficulties.

Proposed Course:

1. The clinical studies in adult ARDs will continue in collaboration with the University of Milan, and is headed by Dr. L. Gattinoni.

2. Laboratory studies will continue to explore treatment protocols in the prevention of HMD and the treatment of HMD through advanced extracorporeal perfusion systems. Hardware will be simplified and the system made easier and more practical to use. We shall explore the proposed mechanism of MAS and evaluate its treatment with extracorporeal CO_2 removal and while on CPAP.

3. The system for total CPBP will be improved on, and smplified. Studies will continue to devise a safe system to allow total cardiac assistance in hearts incapable of left and/or ventricular ejection. This system is expected to become practical for circulatory assist in all patients needing circulartory assistance, including cardiogenic shock.

Publications:

Pesenti, A., Kolobow, T., Buckhold, D., Pierce, J. E., Huang, H., and Chen, V.: Prevention of Hyaline Membrane Disease in Premature Lambs by Apneic Oxygenation and Extracorporeal Carbon Dioxide Removal. Intensive Care Medicine 8: 11-17, 1982.

Kolobow, T., Fumagalli, R., Arosio, P., Chen, V., Buckhold, D., Pierce, J.: The use of the extracorporeal membrane lung in the successful resuscitation of severely hypoxic and hypercapneic fetal lambs. Trans. Am. Soc. Artif. Int. Organs, 1982, in press.

Kolobow, T., Gattinoni, L., Fumagalli, R., Arosio, P., Pesenti, A., Solca, M., Chen, V.: Carbon dioxide and the membrane artificial lung: Their role in the prevention and treatment of respiratory failure. Trans. Amer. Soc. Artif. Int. Organs, 1982, in press.

Uziel, L., Agostoni, A., Pirovano, E., Sedini, I., Pesenti, A., Fox, U., Kolobow, T., and Gattinoni, L.: Hematologic survey during low frequency positive pressure ventilation with extracorporeal CO_2 removal (LFPPV-ECCO$_2$R). ASAIO, 1982, in press.

Kolobow, T., and Gattinoni, L.: The past, present, and the future of the membrane artificial lung. Presented at the International Symposium on Hemoperfusion and Artificial Organs, Ankara, Turkey, Sept. 1982, in press.

Kolobow, T., Gattinoni, L., Solca, M., and Pesenti, A.: A new approach to the prevention and treatment of acute respiratory failure in the adult and the neonate. Presented at the International Symposium of Applied Respiratory Care, Rotterdam, Oct. 1981, in press.

Pesenti, A., Kolobow, T., Solca, M., Buckhold, D. K., and Pierce, J. E.: Prevention of hyaline membrane disease in preterm fetal lambs by apneic "conditioning" of the lungs. ASAIO, 1980.

Kolobow, T., Fumagalli, R., Arosio, P., Chen, V., Buckhold, D., and Pierce, J.: Severe pulmonary capillary alkalosis during states of low pulmonary blood flow: A possible cause of lung damage. ASAIO, 1982, in press.

Pesenti, A., Kolobow, T., Fumagalli, R., Riboni, A., Damia, G., and Gattinoni, L.: Single vein cannulation for extracorporeal respiratory support. ASAIO, 1982, in press.

Kolobow, T., Fumagalli, R., Arosio, P., Chen, V., Buckhold, D., and Pierce, J.: Single vein cannulation for the treatment of acute respiratory failure in preterm lambs with membrane artificial lung bypass. ASAIO, 1982, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01405-08 LTD

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Analysis of Microcirculation by Coherent Light Scattering

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. L. Bowman	Chief, LTD	LTD NHLBI
Other:	R. Bonner	Physicist	BEI DRS

COOPERATING UNITS (if any)
Biomedical Engineering and Instrumentation Branch, DRS

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
This is the final report of this project that provides a monitor of tissue blood flow by analysis of the spectrum of Doppler scattered laser light. The NIH laser Doppler Blood Flow Monitor has been tested clinically as a non-invasive monitor for skin and muscle tissue capillary blood flow. A commercial version of the instrument has been evaluated and found equivalent in performance. Further work on the application of this system will be carried on by Dr. Bonner and associates at BEIB-NIH.

1007

Objectives:

To complete evaluation of the NIH Laser Doppler Blood Flow Monitor in cooperation in clinical and research applications.

Methods:

The NIH constructed model and a commercial product made by Nuclear Pacific Inc., Seattle Washington were evaluated in a number of clinical and reseach applications.

Major Findings:

When some deficiencies in the commercial model were corrected by the maker, the commerical instrument, made under NIH patent agreement was shown to be equivalent. The clinical studies confirmed the convenience, effacacy, and reproducibility of the instrument in the hands of other scientists.

Proposed Course:

Further development is not anticipated in this laboratory. Dr. Bonner at BEIB plans to collaborate with users in the application and further development.

Significance to Biomedical Research and the Program of the Institute:

The NIH Laser Doppler Blood Flow Monitor is now commercially available to make measurements of value to the clinical and research activities for the study of vascular function or diseases. The commerical instrument is manufactured under a NIH license agreement.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01407-19 LTD

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Luminescence Spectroscopy in Biomedical Research

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. F. Chen	Senior Investigator	LTD NHLBI
Others:	D. Mozley	Chemist	LTD NHLBI
	C. Scott	Biologist	LTD NHLBI
	R. R. Hantgan	Assistant Professor	U. of N. Carolina

COOPERATING UNITS (if any)
Dr. Elia Loewenthal, Visiting Professor, RO, NCI

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1. Steady-state fluorescence polarization has been used to detect segmental motions in proteins. Heating of glycerol solutions reveals thermally-activated rotations, while global rotations are obtained in isothermal experiments. The effective size of the rotating unit decreases with temperature.

2. Serum albumin acts as an esterase with certain fluorogenic compounds including acetylsalicylic acid, or aspirin. The rates of reaction have been studied by manual and stopped-flow fluorescence. The results show an unusally reactive site on albumin, which serves as a model for other protein acetylations.

3. Phosphorescence and delayed fluorescence studies of biphenyl and naphthalene in fozen cyclohexane at carbon dioxide temperatures show that diffusion occurs in this solid yet plastic matrix.

1009

Objectives:

The purpose of this project is to investigate problems having importance in the biomedical field using techniques of luminescence spectroscopy. In this way, special luminescence methods are advanced, while simultaneously information is obtained on specific biophysical systems.

Methods Employed:

Chemicals were obtained commercially and purified if necessary by standard methods. Fluorescence polarization data were obtained on an Aminco-Bowman spectrofluorometer, and the large amount of data was analyzed on the N.I.H. DEC-10 computer, largely using MLAB, an interactive modeling language. Stopped-flow and phosphorescence data were obtained with instrumentation already available in the laboratory. No human or animal experimentation was involved.

Description of the Work:

1. Fluorescence depolarization is a simple yet powerful method for determining molecular rotational relaxation rates in the nanosecond range, and was introduced into protein chemistry almost 3 decades ago. Originally the method was supposed to yield the relaxation time of the entire protein ("global" relaxation), which was usually labeled with a fluorescent dye. However, by the mid-1960's, it was clear that the relaxation times obtained were too small, probably reflecting segmental motion, and the method was not considered reliable for global relaxation times. Recently, rapid segmental motions in proteins have been the subject of intense study as the classical picture of proteins as rigid structures has given way to the idea that they are flexible structures in constant motion with rapid segmental fluctuations. Therefore it is appropriate to re-examine the usefulness of fluorescence polarization methods for characterizing these motions.

We have performed steady-state fluorescence depolarization meaurements on protein conjugates as well as protein complexes containing a fluorescent group rigidly bound in a crevice. When such protein systems are heated in solutions whose viscosities differ due to the presence of different amounts of glycerol, segmental motions are clearly demonstrated in classical Perrin plots. Global relaxation rates are obtained in isothermal plots obtained by changing only the viscosity. The heating experiments also show that the effective rotating unit has a volume which decreases with increasing temperature, indicating that rotation becomes less hindered. All of the protein conjugates so far examined show thermally activated rotation of the dye-containing segment, indicating that global relaxation rates cannot be obtained in heating experiments. With rigidly bound fluorescent groups, such as the dye ANS bound to albumin, segmental flexibility is absent and the relaxation times obtained in heating and isothermal experiments are similar. These results are new, because they suggest that many proteins contain thermally activatable segments, and because they define a simple strategy to detect and characterize the segmental motions.

Current work involves comparing the results of steady-state polarization measurements with time-resolved anisotropy data obtained with an ORTEC photon counting system to see if the latter can resolve the presence of several relaxation times.

2. We had previously found that bovine and human serum albumin catalyzed the hydrolysis of fluorogenic esters such as 4-methylumbelliferone acetate, with the probable formation of an acetylated albumin. The reaction can be followed conveniently since the 4-methylumbelliferone acetate is nonfluorescent, while the product, 4-methylumbelliferone is highly fluorescent. Another acetate, acetylsalicylic acid, or aspirin, was found to react much more slowly with the albumins. By determining the reaction rate constants of a variety of fluorogenic esters, it should be possible to define the moecular structural requirements for facile reaction with the albumins.

It has been postulated that aspirin exerts its pharmacological action by acetylating various proteins such as enzymes in the prostaglandin synthetic pathway. Our studies with albumin provide a model for such putative acetylation reactions. So far, it appears that the reaction with aspirin is slow compared with the rates of other acetylators. If aspirin works by acetylation of specific proteins, there must be an unusual affinity exceeding that of albumin for aspirin.

These studies also demonstrate the convenience of fluorogenic substrates and the usefulness of fluorescence stopped-flow kinetics.

3. In collaboration with Dr. Elia Loewenthal, on sabbatical from Hadassah University, we have performed phosphorescence and delayed fluorescence measurements on host-guest systems of interest in radiation biology. Specifically, naphthalene and biphenyl in frozen cyclohexane have been found to exhibit both phosphorescence and delayed fluorescence. Using the Aminco-Bowman in the phosphorescence mode, with a Hg-Xenon lamp, it is possible to detect these luminescences at carbon dioxide tenmperatures. By altering the concentrations of the guest molecules, changing the light intensity (delayed fluorescence arises from triplet-triplet annihilation, a process dependent on the square of the light intensity), we hope to determine the diffusion constant in frozen cyclohexane, which is apparently a plastic matrix. Such studies are in progress, and will help to characterize the plastic matrix for future studies on gamma irradiated samples.

4. We are currently completing projects involving phospholipase activity in lysing liposomes. This work was described in last year's report and has largely been written up and will be submitted shortly. Other nearly finished work involves the energy transfer studies in human serum albumin in which a dye is attached to the single sulfhydryl group, and energy transfer between the dye and the single tryptophan is used to measure the distances between the two groups

5. In collaboration with Dr. R. Hantgan, we have measured the time-resolved anisotropy of fibrinogen labeled with dansyl cadaverine in order to extend steady-state polarization measurements which he has already made. These data provide information on the internal flexibility of fibrinogen, which is important for its biolgoical function.

Proposed Course:

We intend to finish up those projects which are essentially complete and prepare them for publication. Additional collaboration with other laboratories has already started and will be extended; for instance, some work on fluorescent-labeled calmodulin has been started with Dr. Randall Kincaid, CM NHLBI. We intend to apply specialized fluorescence methods to biomedical problems of importance.

Publications:

R. F. Chen: "Fluorescent Dye Leakage from Liposomes to Study Phospholipase ACtivation and Inhibition by Serum Albumin", Abstracts of VII International Biophysics Congress, Mexico City, Aug. 23-28, 1981, p. 159.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01408-17 LTD

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Methodology in Fluorescence Measurements Research

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. F. Chen	Senior Investigator	LTD NHLBI
	G. Liesegang	Staff Fellow	LTD NHLBI

COOPERATING UNITS (if any)
Dr. John J. Pisano, Section Chief, LC, NHLBI

LAB/BRANCH
Laboratory of Technical Development

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INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

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SUMMARY OF WORK (200 words or less - underline keywords)

1. The effect of refractive index (R.I.) of the solution on the fluorescence signal in a spectrofluorometer was analyzed theoretically and experimentally. Although texts state that signal should be proportional to the square of R.I., this was shown to apply only in special cases; in available fluorometers, it is best not to apply correction factor for R.I.'s from 1.3 to 1.6.

2. The fluorometric method for detecting surface-active peptides has been extended to the assay of antibiotics such as gramicidin and polymyxin - indeed to many membrane channel-formers. The method uses liposomes which lyse in the presence of certain peptides, releasing entrapped fluorescent dye.

3. The precision of the steady-state fluorescence depolarization method for analyzing protein relaxation rates depends on photometric precision, the fluorescence lifetime, and the limiting polarization. An expression relating experimental precision to these factors was derived and tested.

4. Storage and analysis of data from a photon counting decay time apparatus has been achieved with an inexpensive microcomputer.

1613

Objectives:

The purpose of this work is to develop and advance new methods of fluorescence assay useful in areas of biomedical research. Attention is also given to the development of standards, the analysis of precision, and the definition of sources of errors.

Methods:

The instrumentation used for our fluorescence measurements has mostly been obtained commercially, but has been modified as described in the past in this laboratory. Experiments do not utilize human or other animal subjects, and the chemicals used are obtained commercially.

Major Findings:

1. Textbooks on physical methods state that the signal obtained from solution fluorescence should be proportional to the square of the refractive index (R.I.). This axiom, accepted by meany, appears to assume a point source of the fluorescence, as well as a geometry such as used in light scattering measurements. We have reviewed the literature supporting this axiom, and find that it ought not to apply to the types of geometry currently used in spectrofluorometers. A concentric cell was fashioned from small and large cuvettes, and the effect of solvent R.I. was tested by filling the outer cuvette with various solvents. With R.I.'s ranging from 1.3 to 1.6, there was no detectable difference in fluorescence signal, indicating that the R.I. -square correction factor was not necessary.

2. Previously, we had developed fluorescent assays based on the lysis of liposomes containing concentrated fluorescent dye (6-carboxyfluorescein). This method is based on the fact that the concentrated dye (0.1 M) is largely quenched through dimer formation, and when the quenching is released as lysis occurs, there is a great enhancement of fluorescence. The assay is useful for determination of phospholipases and for surface active peptides. In collaboration with Dr. John Pisano's group, we have extended the assay to antibiotics such as gramicidin and polymyxin, which may also work through disruption of membranes. In advancing previous work in which we showed that peptides from insect venoms could be monitored as they came off chromatography columns using the fluorescent assay, we have now found a correlation between lysis of the liposomes and mast cell degranulating activity. So far, all peptides showing mast cell degranulating activity are also active in the liposome lysis test, but not vice versa. We intend to write up this work as a useful procedure to determine membrane active peptides.

3. We have analyzed the effect of the length of the fluorescence lifetime on the precision with which protein rotational relaxation times can be measured with the fluorescence depolarization method. The method was introduced for proteins in the mid-1950's by G. Weber and is based on measuring the amount of depolarization which occurs between the

absorption and emission of polarized excitation. For such studies, most workers have used fluorescent dye-conjugates, but our experimental data suggest that most dyes rotate to some degree independently of the protein. Therefore, it would be preferable to use the intrinsic ultraviolet protein emission due to tryptophan. However, many workers in this area feel that the lifetime of protein fluorescence is too short. We have examined this assumption by calculating the amount of error in relaxation time measurements as a function of the length of the fluorescence lifetime. For such calculations it was necessary to have an idea of the photometric precision, which we determined experimentally, and to assume various limiting polarizations. We were able to calculate the fractional error in relaxation time as a function of τ/ρ, the ratio of lifetime to relaxation time. Such calculations had not previously been performed, and they allow certain important calculations: 1. The optimum ratio is about 0.7. 2. For intrinsic protein polarization studies, τ/ρ can be as low as 0.04 for a 10% error in relaxation time. 3. For short relaxation times, τ/ρ may be as high as 10 or 12 before the relaxation time error reaches 10%.

We have confirmed our results with steady-state depolarization data on proteins ranging in molecular weight from 14,300 to 340,000. Global relaxation time measurements were made on proteins with MW as high as 150,000. To do this we depended on isothermal Perrin plots where the viscosity was changed by addition of glycerol. Thus, we have shown that commercially available spectrofluorometers appear capable of determining relaxation times, providing one has a measure of the lifetime.

In contrast, time-resolved, photon counting decay time instrumentation may be less suitable for such measurements. In these instruments, the anisotropy is followed for many nanoseconds following a short light pulse. If the lifetime is short, the anisotropy cannot be followed for a sufficient length of time. Some preliminary experiments of time-resolved anisotropy have been made with Dr. Liesegang which tend to support this idea. Additional experiments are in progress.

4. The ORTEC nanosecond fluorometer is capable of accumulating time resolved fluorescence data, but typically the data reduction systems employed have been inconvenient. Some laboratories have had to store data on tape and physically move them to computer facilities for data handling. We have been able to interface the multichannel analyzer to an inexpensive personal microcomputer (the SOL system) for disc storage and data reduction. Such microcomputers currently cost $2000-3000 and are falling in price. Additional modifications and improvements in the nanosecond fluorometer are in progress.

Significance to Biomedical Research and the Program of the Institute:

Much biomedical research is dependent on fluorometric methods, and advances in these techniques benefit many related fields. The Institute has for many years supported such developments. The current work is consistent with the Institute commitment to further biomedical research.

Proposed Course:

The modification of the ORTEC nanosecond fluorometer will continue with the aim of interfacing it with the MNC computer system which allows more flexibility in data manipulation. Microprocessor-controlled cell changer will be constructed to allow automatic accumulation of blank counts when data are taken over long periods of time. Possible application of picosecond lasers to lifetime measurements will be examined. The depolarization project is virtually complete, but comparisons between steady-state and time-resolved methods should be carried out. The liposome lysis assay method is in the process of being written up.

Publications:

1. R. F. Chen: Fluorescence Spectroscopy: The Refractive Index Corrrection Factor, Anal. Lett. 14(A19), 1591-1601, 1981.

2. R. F. Chen: Citation Classic Commentary. Current Contents 25 INo. 13), p. 20, 1982.

Awards:

Cited by Current Contents as author of a "Citation Classic".

R. F. Chen: Removal of Fatty Acids from Serum Albumin by Charcoal Treatment, J. Biol. Chem. 242, 173-181, 1967.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01411-16 LTD

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Blood Flow Measurement Using Nuclear Magnetic Resonance Techniques

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	V. Kudravcev	Electronic Engineer	LTD NHLBI
	R. L. Bowman	Chief, LTD	LTD NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1	PROFESSIONAL:	OTHER:

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is being discontinued. Current activity has been limited to the evaluation of some recent modifications of the magnetic and electronic circuits to impose a modulated magnetization on a flowing stream and measure flow by picking up the modulated signal down stream where phase delay provides the velocity information.

1017

PHS-6040
(Rev. 2-81)

Objectives:

To evaluate NMR flow methods for application to medical research.

Methods Employed:

We have concluded our project on NMR measurement of the cerebral circulation and now continue to examine some of the techniques for other medical applications. Some instrumentation for modulated nuclear magnetic modulation and detection in flowing fluids was modeled and tested.

Major Findings:

There were no notable advantages over current methods.

Significance to Biomedical Research and the Program of the Institute:

Projects and publications of the NMR research contribute to the general fund of knowledge now being utilized in non-invastive physiological and medical research.

Proposed Course:

No further work is anticipated.

Publications.

Pochobradsky, J.: Optimal Field for Detection of Magnetically Labeled Blood. J. Magnetic Resonance 48: 63-75, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01413-20 LTD

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Instrumentation for the Study of Pre-Steady State Enzyme Kinetics

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. L. Berger	Chief, Sect. on Biophysical Inst.	LTD NHLBI
	P. Smith	Visiting Scientist	BEIB DRS
	G. Liesegang	Sr. Staff Fellow	LTD NHLBI
	P. Bungay	Chemical Engineer	BEIB NHLBI
	M. Perrella	Professor	Univ. of Milan
Others:	C. Gibson	Electrical Engineer	BEIB DRS

COOPERATING UNITS (if any)
Biomedical Engineering and Instrumentation Branch, DRS

LAB/BRANCH
Laboratory of Technical Development

SECTION
Section on Biophysical Instrumentation

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 2	PROFESSIONAL: 1	OTHER: 1

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A small, easily constructed quench flow apparatus has been constructed with time resolution for cold quenching of 5 milliseconds. Improvements in the ball mixer design have made this possible. A dual wavelength laser flash photolysis system has been built and tested on carbon monoxide hemoglobin at a concentration of 4 mM in heme. 50% fast reacting hemoglobin is still obtained.

1019

PHS-6040
(Rev. 2-81)

Objectives:

The objectives of this project are to develop new instrumentation methods, data handling techniques and theoretical treatments for the physiochemical study of the thermodynamics, kinetics and thus the mechanisms of enzyme action in solution and in the intact cell or cell membrane. In particular, to develop methods and instruments to study, in collaboration with other laboratories, the reactions of hemoglobin with the respiratory gases both in normal state as modified by the changes of physical factors, small molecules, various metabolites, and genetically, such as in sickle cell anemia. The reactions of various cellular enzymes, such as ATPase, calmodulan, their interactions and control in the cell are studied as they relate to cardiology, pulmonary and respiratory function, and circulation. Where appropriate analytical methods are developed for research and clinical application.

Methods Employed:

The methods used in the investigation of the mechanisms of enzyme action are those of pre-steady state chemical kinetics and thermodynamics. Measurements of the appropriate parameters are made by developing the necessary equipment to mix solutions rapidly and follow the course of the resulting chemical reaction by optical, thermal, glass electrode, etc., detectors. In general, equipment is not available, either in the literature or commercially for investigations in this area. Such apparatus is conceived and designed in this laboratory, together with consultants, construction being carried out wherever most appropriate; i.e., in our shops or by commercial firms, special university facilities, or at several special research laboratories such as the Jet Propulsion Laboratory. In pursuing these investigations, a wide variety of physical parameters must be studied, which leads to the need for an understanding of the underlying physical theory governing the reactions. Expert consultants and collaborators are brought in to assist in the design, analysis, and evaluation of the equipment, particularly as it is applied to certain specific enzyme systems under investigation.

Major Findings:

The development of an improved thermal stopped flow apparatus has continued in conjunction with Bungay in BEIB who is measuring pressure drops in various mixer configurations, to develop a better theoretical understanding of turbulent mixing in the ball mixer. Lack of trained personnel in BEIB and the use of students has greatly slowed this project.

On the other hand, at the request of Perrella in Milan and Rand in Canada we have used the information gained from the mixer study thus far to design a simple but fast quenching system for the study of enzyme-substrate reactions in calcium membrane interaction studies and hemoglobin intermediate isolation. A new design for a very simple, but highly reproducible stepping motor syringe drive with very high acceleration has been designed and a proto-type is presently being constructed.

The laser flash dual wavelength apparatus has been completed and successful experiments conducted on CO Hb reactions at cellular concentrations. Results indicate 50% fast reacting Hb still exists even at 4 mM heme. This is in apparent agreement with a new model of Hb action recently proposed by Ackers (Johns Hopkins Univ) at a recent Fogarty Center Lecture. Work with whole cells has been dissapointing but it is possible that the solution must be flowed at a rate fast enough to randomize the cell orientation.

Proposed Future Work:

A new drive system will be tested and, if successful incorporated into the thermal stopped-flow. Considerable testing will be needed to prove out newly designed mixers and equilibrators.

Signficiance to Biomedical Research and the Program of the Institute:

The study of the kinetics of chemical and enzyme-substrate reactions is a widely applied field yielding important information for interpreting the mode of action of the chemical or enzyme under study. Continued development of the response and the sensitivity of different sensor techniques, eg. thermal, pH, and optical methods, enhances the ability for an investigator to explore with finer detail a system of interest. The particualr reactions chosen to test the capability of the instrumentation being developed are of immediate significance: binding of calcium by EGTA is important for studying muscle contraction kinetics, studying hemoglobin-ligand binding studies provide information for understanding the complex action of this molecule.

Publications:

1. Balko, B., Berger, R. L., and Anderson, K.: Measurement and simulation of thermistor response time in the millisecond range. Rev. Sci. Instrum. 52 (6), pg. 888, June 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01414-10 LTD

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Development of Microcalorimeters and Differential pH Thermal Titration Apparatus for Biochemical Reaction Studies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. L. Berger	Chief, Sect. on Biophysical Inst.	LTD NHLBI
Others:	C. Mudd	Mechanical Engineer	BEIB DRS
	W. Friauf	Chief, Electrical Engineering Sect.	BEIB DRS
	M. Marini	Professor Biochemistry	N.W.U. Med. School

COOPERATING UNITS (if any)

Biomedical Engineering and Instrumentation Branch, DRS

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SECTION

Section on Biophysical Instrumentation

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 3	PROFESSIONAL: 1	OTHER: 2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

An electric circuit analog of the batch calorimeter has been set up and analyzed by La Place Transform which greatly assists in the design of the calorimeter and in predicting the dynamic response of the instrument. The D-B Finite Element Simulation Technique (FEST) was used to data correct the output of the calorimeter permitting a reaction with half-life one-one hundreth of that of the calorimeter to be measured. A differential pH-thermal apparatus has been constructed and tested. It is being used to study calcium binding to EGTA, phytic acid and calmodulin.

1022

PHS-6040
(Rev. 2-81)

Objectives:

An understanding of the fundamental mechanism of biological reactions requires three physical factors: a) the kinetics, i.e., pathway of the chemical reaction, b) the thermodynamics of the reaction, i.e, what energy is available and how does it change from one state to another,and c) the stereochemistry of the reactants. This project addresses these factors by developing appropriate instrumentation to study heat changes which will give a deeper insight into the mechanism of the relevant biochemical reactions involved in the functions of enzymes and cells. Model reactions are chosen to demonstrate the effectiveness of the methods.

Methods Employed:

Initial designs are constructed in this laboratory with special assistance from commercial firms in the construction of sensors; contracts are let, where warranted, for the development of completed instruments with refinements that would tax our own facilities. The instrument is then tested in conjunction with other interested biochemical calorimetrists utilizing appropriate enzymatic and cellular reactions.

Major Findings:

Automation of both the batch calorimeter and the pH-thermal titration apparatus have led to the recognition of a number of interesting problems. The batch calorimeter we have designed has been analyzed both by the D-B Finite Element Simulation Technqiue (FEST) and also by the classical electrical analog method using LaPlace Transforms. This has provided much improved design data and a data correction scheme that permits the kinetics of reactions 100 times faster than the response of the calorimeter to be studied. This required an improvement in the data reduction system. As a result 12 bit A/D converters have been replaced by 16 bit A/D's. A clear need for this was shown by a computer experiment using FEST. At the same time the electronic network analog method showed clearly the error in rise time if the air space between the calorimeter sample cell and copper block was not kept constant to ± .0005 inches. A greatly simplified method of holding the cell is presently under test which should eliminate this problem.

The pH-thermal titration apparatus testing program showed up very interesting problems in feed back control of titrant addition and simultaneous data collection. While we thought this could be done by software, the time frame involved was simply too short. We have taken advantage of a recent improvement in real time clock boards and have added a 16 bit A/D converter plus clock board to our S-100 bus automation computer. Thus all actual control functions are set and run by hardware, the software only starting, stopping or changing speeds. In addition to testing with standards, i.e. KOH + HCl reaction and histidine, titrations (combined pH-thermal) have been carried out on phytic acid, EGTA, and, presently in progress, calmodulin. The pK's of titratable groups, and their shifts on the binding of Ca, have been measured as well as the heat of binding . The binding constants of Ca can also be obtained. Table Ia and Ib summarizes the data.

TABLE Ia

HISTIDINE

Groups	pK^1	±	ΔHi	±	ΔH_{H2O}
1	1.80	0.30	1.18 Kcal mol^{-1}	0.01	13.7 Kcal mol^{-1}
1	6.01	0.02	7.36 kcal mol^{-1}	0.07	
1	8.93	0.02	10.61 Kcal mol^{-1}	0.08	

LITERATURE VALUES

pK	ΔHi
2.14	kcal mol^{-1}
6.08	7.4 Kcal mol^{-1}
9.12	10.7 Kcal mol^{-1}

TABLE Ib

CALCIUM/PHYTIC ACID 6:1 MOLAR RATIO

GROUPS	pK'	ERROR	ΔHi	ERROR
5	1.34	0.11	-3.46 Kcal mol^{-1}	0.47 Kcal mol^{-1}
1	2.24	0.22	-3.46 Kcal mol^{-1}	0.47 Kcal mol^{-1}
5	5.30	0.02	3.16 Kcal mol^{-1}	0.06 Kcal mol^{-1}
1	7.92	0.03	8.67 Kcal mol^{-1}	0.25 Kcal mol^{-1}

Proposed Course:

A new differential calorimeter will be tested and applied to a number of biochemical reactions. In addition, an attempt will be made to operate it in a scanning mode to study the intra-molecular forces in the folding of proteins. Work will continue on the flow microcalorimeter and the differential pH apparatus.

Significance to Biomedical Research and the Program of the Institute:

An understanding of the basic mechanisms by which enzyme catalyzed reactions carry out the vital processes of life is of fundamental importance. A necessary part of this understanding is provided by a measurement of the thermodynamics of the reaction. This will ultimately lead to enhancement of our ability to prevent and treat the heart, lung, and blood ills the institute is charged with.

Publications.

Davids, N., and Berger, R. L.: Computer simulation for deconvolution of a heat conduction batch microcalorimeter by the D-B finite element technique. J. Biochem. Biophys. Methods, 1982, in press.

Mudd, C., Berger, R. L., Hopkins, H. P., Friauf, W. S., and Gibson, C.: An optimized differential heat conduction solution microcalorimeter for thermal kinetic measurements, J. Biochem Biophys. Methods, 1982, in press.

Berger, R. L, Balko, B., Clem, T. R., and Friauf, W. S.: Fast thermistor sensors for rapid raction studies, Instrument Society of America, 1982, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01415-09 LTD

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Italy - U. S. Cooperative Science Program - Blood Gas Instruments, Project 78

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. L. Berger	Chief, Sect. on Biophysical Inst.	LTD NHLBI
	L. Rossi-Bernardi	Professor of Biochemistry	U. Milan
	T. Clem	Electrical Engineer	BEIB DRS
	C. Gibson	Electrical Engineer	BEIB DRS

COOPERATING UNITS (if any)
Biomedical Engineering and Instrumentation Branch, DRS
University of Milan, School of Medicine, Milan, Italy

LAB/BRANCH
Laboratory of Technical Development

SECTION
Section on Biophysical Instrumentation

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 4	PROFESSIONAL: 2	OTHER: 2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
A new oxygen equilibrium curve apparatus has been undergoing extensive testing and debugging.

1026

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

An understanding of the various physical factors that affect the delivery of oxygen to the tissues and the removal of CO_2 are vital to a correct interpretation of the physiological response of the human body to various disorders, disease states, and environmental stress. As part of a long range basic research program being carried out in collaboration with the School of Medicine, University of Milan, the Hematology Division, Bureau of Laboratories, CDC, and the Cero dePasco High Altitude Research Laboratory, Universidad Peruana Cayetacho Heredia, Lima, Peru, we are engaged in the development of highly accurate instrumentation to measure the oxygen dissociation curve in whole blood and concentrated hemoglobin solutions, the hemoglobin components, in whole blood, Hb, Hb+, HbCO, and HbO_2, the pH, pCO_2, and PO_2, and the concentration of 2-3 DPG. Ultimately we hope to measure the tissue pO_2 and PCO_2 levels at the mitochrondia cytochrome oxidase site.

Methods Employed:

A systematic analysis of the complex interrelationships among several variables and the effect on the oxygen dissociation curve requires the development of a method to obtain oxygen dissociation curves of human blood in vitro, under conditions closely simulating the in vivo situation of the patient. Instruments are developed either at NIH and/or Milan, tests on pure hemoglobin are generally conducted first in Milan, where a large group is currently working on the purification of hemoglobin.

Testing on patient blood is then carried out at the Red Cell Disease Branch, CDC and at Cero de Pasco, Peru. Close cooperation exists with the medical school hospital in Milan where on-line computer monitoring will be carried out using the membrane oxygenator system developed in this laboratory by Dr. Theodor Kolobow.

Major Findings:

The oxygen Equilibrium Curve (OEC) Apparatus has been undergoing extensive hardware and software debugging. As a result, a detailed analysis of the operation of the system indicated that major changes in both the hardware and software of the microprocessor would need to be changed if rapid real time response to changes in the PCO_2 due to the release of protons by hemoglobin as it is oxygenated. NaOH is added in response to these changes, sensed by the PCO_2 electrode in the OEC apparatus cell. Since all of this work has had to be done by coop students due to the lack of trained personnel, it has gone rather slowly. All in all this has probably been a helpful event in that their coming to the instrument totally ignorant of how it works, or why, has forced a much more careful explanation of the system resulting in overall improvements suggesting themselves during the explanation.

Proposed Course:

The use of glutathione peroxidase (Selenium dependent) will be tried to see if H_2O_2 can be reduced to oxygen fast enough to avoid oxidizing to methemoglobin the deoxyhemoglobin in solution. Considerable futher testing of this instrument and a diffusion membrane system will be done in an effort to provide scientists studying variant hemoglobins with a reasonably fast, highly accurate instrument for measuring the equilibrium curve in both cells and solutions.

Significance to the Program of the Institue:

An understanding of the factors controlling the delivery of oxygen and the pickup of CO_2 in the tissues as well as their subsequent movement across the alveolar is of fundamental importance to both the prevention and of heart, lung, and blood disease.

Publications:

1. Berger, R. L., Clem, T.: Fast thermisotrs in Temperature, Its Mesurement and Control in Science and Industry, ISA, 1982, in press.

2. Winslow, R. M., Monge, C. C., Statham, N.J., Gibson, C. G., Charache, S., Whittembury, J., Moran, O., and Berger, R. L.: Variability of oxygen affinity of blood: humasn subjects native to high altitude. J. Appl. Physiol. 51 (6), 1411-1416, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01421-07 LTD

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Development of Electrochemical Methods for Kidney Research and Blood Analysis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. Steele	Physical Scientist	LTD NHLBI
Others:	V.A. Hampshire	Physical Sci. Techn.	LTD NHLBI
	J. Handler	Section Chief, LKEM	LKEM NHLBI
	A. Preston	Chemist	LKEM NHLBI
	W. Haller	Laboratory Chief	NBS

COOPERATING UNITS (if any)
Laboratory of Kidney and Electrolyte Metabolism, NHLBI

LAB/BRANCH
Laboratory of Technical Development

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INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 2	PROFESSIONAL: 1	OTHER: 1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Work on methods and apparatus for the study of kidney epithelial cells grown as sheets on porous membranes continues to yield important results. The central element is the porous bottomed culture dish (PBCD) in which the membrane is cemented to polycarbonate rings which provides electrical and chemical isolation between the solutions on each side of the membrane. With these PBCDs new knowledge of the development of epithelial is being obtained. The increased exchange at the basal side (blood side) leads to a vasopressin response for A6 cells (from Xenopus laevis kidney) which is otherwise absent. Transparent collagen membranes on the PBCDs have allowed these devices to be used effectively for several primary cultures as well as established lines. The importance of Ca^{++} activity in the control process of epithelial cells has resulted in a project aimed at improving the selectivity of Ca^{++} electrodes. Improvement is particularly needed for electrodes with tip diameters <0.1 microns so that activities less than 10^{-6} molal can be measured in epithelial cells.

1029

PHS-6040
(Rev. 2-81)

Objectives:

(1) Develop porous bottomed culture devices with special properties:

A. Transparent membranes
B. Membranes thin enough for microscopy and microelectrode work
C. Small enough for slow growing cells
D. Very small for growth from one cell
E. Membranes incorporating type IV collagen and laminin to facilitate the attachment of epithelial cells.

(2) Design sterile continuous flow apparatus for study of epithelial cells layers on transparent membranes so that the development of transport and morphology can be studied for weeks or even months.

(3) Develope electrodes to measure Ca^{++} activity in epithelial cells as they develop their transport capability.

(4) Develope a differential $_pO_2$ electrode system for the measurement of oxygen consumption by epithelial cells growing on porous membranes.

Methods Employed and Major Findings:

Porous bottomed culture dishes, PBCDs, have been constructed with 1, 2, 5, 6, 11 and 38 millimeter diameter collagen membranes. These have been useful for observing cells as they grow on these transparent membranes. Cells from the kidney of the Xenopus laevis (established line A6) were placed on a 5, 6 and 38 mm collagen membranes and developed transepithelial potential differences (PDs) of about 20 millivolts which were maintained for as much as 8 months.

It has been shown that the A6 cells require basolateral feeding in order to differentiate in such a way that they respond to vasopressin. Basolateral feeding cannot occur when the cells are grown on the usual tissue culture pettri dishes and these 6 cells never show vasopressin response immediately after being removed from the dish suface as indicated by adenylate cyclase activity. When grown on PBCDs with Millipore filter bottoms these A6 cells have always shown a vasopressin response. When grown on PBCDs with collagen coated Nuclepore bottoms these A6 cells usually show a vasopressin response only if the PBCDs have feet which space the porous membrane about 1 mm above the bottom of the containing dish in order to increase basolateral exchange. This result with the collagen coated Nuclepore filter material is not always reproducible prbably due to low permeability of this composite membrane. This low permeability probably also accounts for the large changes in PD, electrical resistance and short circuit current which are observed after medium changes or stirring.

The PBCDs allow the media and PD to be different on the 2 sides of the membrane. At present the PD across the membrane is that which the cells produce. This is similar to what would occur in the animal as it develops. Until recently the same medium (including serum additive) has been used on both sides; however, in order to control cell growth (to avoid multilayers of cells) the apical medium in some experiments has been replaced by serum free medium. This did give some growth control. In some cases it also gaves PDs which started up sooner and went higher. On collegen membrane PBCDs it could be seen that the movements and shape changes of the cells were simplified and they went more directly to the final form when the serum free medium was put on the top when the cells were first confluent. It appears that we have some very useful tools with which to study the basic developmental processes of epithelia.

Dr. John Forrest from Yale (on Sabbatical in LKEM) has recently grown primary cultures on PBCDs with 5 mm collagen membranes. They were cells from the papilla of the rat kidney and the urinary bladder of the toad (Bufo Marinus).

The importance of calcium to the control processes of epithelial cells has caused us to work on improving the selectivity of ion specific Ca^{++} electrodes. The present techniques for measuring Ca^{++} activity inside cells, dyes and electrodes, both give results which are probably too high. The dyes indicate to high an activity because the ones that work at all have Ca^{++} affinities which are comparable to some of the things inside the cells which bind Ca^{++}. The electrodes read too high due to interferences from other ions: Na^{+}, K^{+}, Mg^{++}, etc. These interferences are particularly troublesome when the electrode tips are made 0.1 microns or less in diameter. Such tip sizes are necessary to make stable measurements inside epithelial cells. The changes being explored to obtain improved specificity are:

(1) Use of glasses without diffusable ions such as fused quartz and Corning 1723.

(2) Silanization of the glass tips with monochlorosilanes with long hydrocarbon chains and with careful exclusion of water vapor.

(3) Explore attachement of the Ca^{++} exchange molecules directly to the glass.

It is hoped that these efforts will result in electrodes with tips smaller than 0.1 microns which can accurately measure Ca^{++} activities of less than 10^{-6} molal which probably exists in most cells.

Significance to Biomedical Research and the Program of the Institute:

The apparatus and methods necessary to grow and study sheets of epithelial cells on membranes is advancing the study of the basic mechanisms of active Na transport. The ability to make the measurements under sterile conditions greatly increases the productivity of this work. Dr. Handler, LKEM, NHLBI, is presenting the physiological significance of this work in detail. The growth of the cells on nutrient and gas permeable membranes results in a degree of development and differentiation which does not occur on the conventional plastic or glass surface. This should allow a study of the development of epithelial that is not possible otherwise. The accurate measurement of the Ca^{++} activity inside the epithelial cells at various stages of transport development should greatly increase our knowledge of transport development and control.

Proposed Course:

(1) Design sterile continuous flow apparatus for sterile study of sheets of epithelial cells on transparent membranes for weeks or even months so that the development of transport and morphology can be followed.

(2) Develop very small collagen PBCDs which can be seeded with only 1 cell. The feeder layer often required in cloning can be provided on the other side of the membrane. The feeder cells can grow either in the bottom of the dish holding the PBCD or right on the membrane (planted previously with PBCD upside down).

(3) Develope electrodes to measure Ca^{++} activity in epithelial cells which have tips smaller than 0.1 microns and low interferences so that Ca^{++} activities of 10^{-6}, 10^{-7} and even 10^{-8} molal can be measured correctly.

Publications:

1. Handler, J. S., and Steele, R. E.: Epithelial function in clutured epithelial cells. The kidney during development - morphology and function, Edited by A. Spitzer, 67-74, 1980, Masson, New York.

2. Johnson, J. B., Steele, R. E. Perkins, F. M., Wade, J. B., Preston, A. S., Green, S. W., and Handler, J. S. : Epithelial organization and hormone sensitivity of toad urinary bladder cells in culture. Am. J. Physiol. 241 (Renal Fluid Electrolyte Physiol.). F129-F138. 1981.

3. Burg, M., Green, N., Soharaby, S., Steele, R. E., Handler, J.: Differentiated function in cultured epithelia derived from thick ascending limbs, Am. J. Physiol. (Cell), 1982, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01435-03 LTD

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

New Catheter Idea to Facilitate Radiologic Instrumentation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: R. L. Bowman Chief, LTD LTD NHLBI

COOPERATING UNITS (if any)

Diagnostic Radiology Department, Clinical Center, NIH

LAB/BRANCH

Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The concept of topocatheter extension to the standard angiographic catheter to facilitate entry into small and tortuous vesels involves the production of a high strength thin walled tube that can be easily turned inside out. When the thin wall extension is exposed to negative pressure inside the catheter it first collapses and then inverts itself until it lines the distal end of the catheter. pressure inside the catheter causes the lining to form a valve and shut so that flow can only occur by again inverting the thin wall tube. When the thin wall tube everts it rolls onto the inside of the vessel wall with no friction so that it can enter tortuous vessels with ease. A 12 cm extension on a No. 7F catheter tapered to 1mm is typical. A triangular tapered shape reduces self inteference of the thin wall extension.

1033

Objectives:

To continue to develop ideas to improve the performance of angiographic catheters. Current activity involves the development of an extension to a conventional angiographic catheter to reach smaller or enter tortuous vessels.

Methods Employed:

The catheter extension developed previously have been limited in length and size by the limitation on flexibility imposed by the materials constructed so that there is still a need for more compliant materials that can be securely attached to the primary catheter.

Several improvements introduced last year included a semiautomated apparatus for drawing polyurethane ester or ether, a triangular cross section to facilitate inversion and drawing large 3/16 I.D. tubes to facilitate mounting on catheters.

The need for lubrication, a satisfactory cement and a better source of drawing stock have been solved by the development of a satisfactory system of producing thin walls, triangular shapes and the selection of a stock made by Stevens. The use of a polyurethane primary catheter permits solvent welding the extensions.

Polyurethane stock in the form of 3/16 x 5/16 tubing in the form of polyether or polyester obtained from Stevens is mounted in a pair of chucks concentrically and synchronously driven by a chain drive at about 33 RPM. The rotating material is heated in the focused beam of a projection lamp DLG 150W 21V and run at reduced voltage. A projection lens produces an enlarged image of the heated region on an observation screen to facilitate selection of the time to pull the chucks apart in line while still rotating. Either 3 internal berylium copper fingers or dag dispersion No. 33 in the form of three stripes modifies the heat pattern to produce a triangular form of the drawn product.

A motor driven drawing motion provides reproducible conditions to permit a reasonable yield ca 10% of useable extensions with a 75 second heating period before drawing.

Catheter extensions are tested for defects largely related to defects in the stock tubing and selected for size and taper which varies for unknown reasons.

Testing is carried out in a simulated circulatory system against arterial pulsating pressures and visualized by simulated contrast materials utilizing Rhodamine solution and UV light to illuminate the field.

Glass models of complex arterial shapes are provided to challenge the catheters ability to enter tortuous vessels while injection of Rhodamine fluorescing in the UV light indicate the location of the catheter similar to the way it is visualized by contrast material introduced for X-ray visualization.

In addition this year the extensions have been tested in the dog circulation.

Number 7 polyurethane intrarterial catheters have been produced with up to 19 cm extension with terminal diameters of 1 mm have been tested on the simulator.

Additional methods of fabrication included the double walled version developed previously but difficulty of construction made this approach unattractive and was not further used this year..

Construction by dip coating materials while successful at times was not as versatile when drawn glass mandrilles were used as even coatings free of defects were not easily produced. Dip coated rubber latex was also used but the thickness and strength were not satisfactory.

More recently we have coated glass or stainless steel mandrills with collagen. Mandrills were rotated while coating with solution of collagen and when dry more collegen was applied until it built up to approximately .001 inch wall thickness.

Major Findings:

Satisfactory catheter extension could be made with about 10% yield. Reconstituted collagen in the first few cases produced nearly ideal catheters with the proper lubricity and all the properties required to extend and retract.

Significance to Biomedical Research and the Program of the Institute:

While these preliminary tests have been most encouraging it remains to be proven that we can reproduce them with a tolerable yeild. It would also appear that the use of collegen that has already been accepted for medical use would facilitate its acceptance for human use.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01440-02 LTD

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Microfluorimeter for Analysis of Femtomoles Amounts of Biochemicals

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. G. Vurek	Senior Investigator	LTD NHLBI
	D. Good	Guest Worker	LKEM NHLBI

COOPERATING UNITS (if any)
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LAB/BRANCH
Laboratory of Technical Development

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NHLBI, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project involves the development and testing of a new fluorimeter with a working volume of less than 300 nL. The fluorimeter is part of a flow through chemical analysis system. An optical fiber brings light from a filtered mercury light source and carries the light to the cuvette. The tip of the fiber is in the axis of the cuvette; fluorescent materials flowing past the tip are efficiently excited, and the resulting fluorescence is measured by a nearby photomultiplier. The system can detect femtomole amounts of highly fluorescent materials and picomole amounts of ammonium ions with the aid of standard enzymic assay chemistry.

1036

PHS-6040
(Rev. 2-81)

Methods Employed:

The cuvette was made of Suprasil quartz tubing, a material of low intrinsic fluorescence, about 0.2 mm i.d. x 0.5 mm o.d. This was bent into a sort of contorted hairpin shape using microglassworking techniques; a small hole was blown at one portion of the tube so that the end of an optical fiber could be inserted into the lumen of the tube and sealed in place. The other end of the fiber was placed adjacent to a glass filter that transmits the longwave ultraviolet wavelengths emitted by a 100 watt mercury lamp. The cuvette was mounted close to the cathode of a photomultiplier tube; a piece of gelatine filter which absorbed the stray excitation light was between the cuvette and photomultiplier.

Since the volume of the fluorometer is less than 0.3 microliter, a small reservoir for reagents is used. In the present model, the reservoir is a pasteur pipette. Reagent flows from the pipette to an injection port where samples for analysis are injected. The present design of the port is a simple tube with a small hole in the top surface; surface tension holds the reagent in place. Samples are injected into the flowing reagent stream using a nanoliter transfer pipette. The sample and reagent are drawn through the cuvette by a syringe-type withdrawal pump operating at a rate of 110 nL/sec. The intensity of fluorescence as a function of time is recorded and the peak fluorescence after a sample is injected is used to quantitate the amount of sample injected. The fluorimeter is currently being used to assay the amount of ammonium ions produced by isolated rat kidney tubule segments.

Major Findings:

Tests using continous streams of various concentrations of a highly fluorescent material, 4-methylumbelliferone, showed that the fluorimeter would be able to detect the presence of 6×10^{-15} moles of that material. When 11 nL samples of various amino acids were injected into a reagent stream made of phthalaldialdehyde and dithiotreitol, the fluorimeter could detect less than 1/2 picomoles of l-glutamic acid and others. Ammonium ions produced by rat kidney tubule fragments at the picomole level have been measured using a reagent stream containing alpha-ketoglutarate, NADH, and glutamic dehydrogenase. Using this new fluorimeter and technique, it has been possible to show that the site of significant ammonium production is in the proximal straight segments of the rat kidney tubules.

Significance to Biomedical Research and the Program of the Institute:

The new nanoliter flow-through fluorimeter opens up a wide range of single cell and tissue fragment studies for biochemical assays. In addition, the system offers a detector for HPLC that is superior to existing detectors.

Proposed Course:

The basic principles and performance of the system have been established. While other improvements could be made, such a sample size reduction and adjustable wavelength, these will not be pursued in the near future. A complete description of the ammonium ion assay will be published.

Publications:

Vurek, G. G.: Nanoliter flow-through fluorometer. Anal. Chem. 54: 840-842, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01443-01 LTD

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Miniature Sealed pH Electrode

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: G. G. Vurek Senior Investigator LTD NHLBI

COOPERATING UNITS (if any)
Anesthesia Research Group, CVRI, UC San Francisco, CA

LAB/BRANCH
Laboratory of Technical Development

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INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A miniature pH electrode was fabricated with a glass-to-metal seal between the glass electrode body and the silver of the internal reference electrode. The electrode was about 1 mm in diameter and 5 mm long. It had a room temperature sensitivity of 55 mV/pH. One such electrode was made part of a miniature carbon dioxide sensor.

1039

Methods Employed:

The electrode was fabricated from Corning Glass type 0120 glass (pH sensitive), type 0150 glass (insulating glass), and silver wire. Using a miniature gas/oxygen torch with a highly oxidizing flame, it was possible to fuse one end of a 1 mm dia. tube of 0120 glass to the silver wire despite the large difference in expansion coefficients of the materials. Then a length of 0150 glass could be slipped over the silver wire and fused to the 0120 glass. The tip fo the 0120 glass opposite the glass-to-metal seal was drawn out and the interior space was filled by gentle heating and cooling with the open tip immersed in buffer. When there was some buffer inside the electrode, the tip was carefully sealed so that the heat from the flame did not evaporate the internal buffer or cause the electrode to explode. After subjecting the electrode to a seal integrity test, the lead wire was covered with silicone tubing which slipped inside the 0150 insulating glass. The space around the lead wire in the silicone tube was filled with silicone glue, which also filled the space between the 0150 glass and the tube.

Major Findings:

Silver wire smaller than 100 micrometers in diameter seems to be sufficiently malleable to accommodate the thermal strains associated with a glass-to-metal seal between it and 0120 glass. This means that the inner electrolyte of a sealed pH electrode can be completely isolated from the influence of external materials. Other electrodes of these dimensions have been been filled with polymeric substances. When tested with standard buffers, the sealed electrode showed a slope sensitivity at room temperature of at least 55 mV/pH and a long term drift of less than 10 mV (six weeks). One electrode was incorporated into a miniature carbon dioxide sensor. When tested, it showed appropriate response to changes in the partial pressure of carbon dioxide.

Significance to Biomedical Research and the Program of the Institute:

The technique of making completely sealed pH electrodes offers a way to produce electrodes wiht good long term stability and good resistance to the effects of immersion or implantation. This may lead to implantable sensors with valuable properties for physiologic research.

Proposed Course:

No further work is planned at this time.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01444-01 LTD

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Fiber Optic Carbon Dioxide Sensor

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. G. Vurek	Senior Investigtator	LTD NHLBI
Others:	P. J. Feustel	Research Physiologist	CVRI, UCSF
	J. W. Severinghaus	Director, Anesthesia Research Group	CVRI, UCSF
	J. I. Peterson	Chemist	BEIB DRS
	S. R. Goldstein	Mechanical Engineer	BEIB DRS

COOPERATING UNITS (if any)
Anesthesia Research Group, CVRI, UC San Francisco, CA
Biomedical Engineering and Instrumentation Branch, DRS, NIH

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Laboratory of Technical Development

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INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A new optical sensor for carbon dioxide has been developed The sensor is based on the principle that pH of a bicarbonate solution in equilibrium with gaseous carbon dioxide can be estimated by measuring the light transmittance of an indicator dye. A flexible probe about 60 cm long with a sensitive tip was made using two plastic optical fibers. One carried light from a filtered incandescent lamp to the probe tip and the other returned light that had passed through the phenol red dyed buffer to a photosensor. The tip is about 0.6 mm dia. and 0.8 mm long. Tests show good linearity over the range from 20 to 100 mm Hg and a 95% response time of one minute.

1041

Methods Employed:

The purpose of this study was to develop an optical technique for measuring carbon dioxide tension and to demonstrate its value in estimating the partial pressure of carbon dioxide in the fluid surrounding the medullary respiratory control center of the cat. The approach was to take a small piece of silicone rubber tubing, permeable to carbon dioxide gas but not to ions, containing about 0.3 microliter of potassium bicarbonate/phenol red buffer. The light absorbance at 570 nm is a function of the ambient carbon dioxide tension. The sensor was made by taking two 125 micrometer dia. plastic optical fibers and putting them in a 60 cm long black plastic sheath so that the tips protruded from one end about 0.5 mm and from the other about 20 cm. The silicone tube with the dyed buffer was placed over the end of the sheath with the short stubs and sealed. The long ends were separately sheathed; one was put at the focus of a beam of light from a tungsten-halogen lamp that had passed through one of two filters. One filter, an interference filter with a peak transmittance at 570 nm, passed light strongly absorbed by the base form of the dye. The other filter passed red light not absorbed by the dye. The other fiber terminated at the sensitive region of a phototransistor. Its output drove a transconductance amplifier with a gain of 10 g volts/amp.

Major Findings:

Theory shows that this sort of sensor can be made to give a linear response over the physiologically useful range from 20 to 100 mmHg. The probe was tested in vitro by exposing it to humidfied gases of various compositions. Over the full range from 0 to 700 mmHg, the response was sigmoidal as predicted, and the response in the physiologic range was linear within 5% as predicted. The response showed a -3.2%/degree shift in slope, also expected by gas solubility considerations. The time to achieve 95% of the steady state response to a step change in carbon dioxide partial pressure is one minute. In vivo tests comparing the optical probe respone to standard blood gas measurements for changes in arterial blood gas tension showed correlations greater than 0.99 with a mean difference less than 3 mm Hg. The probe was also tested by placing it on the cortex of a cat and covering the probe with a transcutaneous carbon dixoide sensor. Again the correlation was excellent, with a difference of less than 4 mm Hg.

Significance to Biomedical Research and the Program of the Institute:

The probe offers a way to study tissue carbon dioxide tensions without the necessity of a fragile, unstable pH electrode system of the sort that has been used heretofor. The probe offers a small, lightweight flexible form with good long term stability and requires no electrical connection with the subject.

Proposed Course:

The probe described here is continuing to be evaluated as a sensor in a study of the tissue-arterial blood carbon dioxide gradient in the vicinity of the medullary respiratory control center of the cat. Further probe development will be carried out to test forms with a single small fiber so that the sensitive zone can be reduced to as small as 50 micrometers in diameter.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01445-01 LTD

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Development of Multi-Layer Coil Countercurrent Chromatography (CCC) for High Speed Preparative Separations

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Y. Ito	Medical Officer	LTD NHLBI
Others:	Jesse Sandlin	Volunteer Worker	LTD NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Technical Development

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NHLBI, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A newly developed countercurrent chromatographic (CCC) scheme yields a highly efficient preparative-scale separations in a short period of time ranging 2-5 hours. The method uses a coil planet centrifuge which produces a synchronous planetary motion of the holder. The column consists of multiple layers of the coil called "multi-layer coil", which is accommodated on a spool-shaped column holder. The system produces excellent retention of the stationary phase against a high flow of the mobile phase. Consequently, solutes locally introduced at the inlet of the column are efficiently separated according to their partition coefficient in the manner analogous to the liquid partition chromatography but in the absence of solid-supports. The capability of the present method has been demonstrated on separation of various biological materials which include DNP amino acids, natural and synthetic peptides, indole plant hormones, purines and pyrimidines, etc.

1044

PHS-6040
(Rev. 2-81)

Methods Employed and Major Findings:

a) Principle:

The method utilizes a complex hydrodynamic motion of two immiscible solvent phases in a coiled tube undergoing a synchronous planetary motion. As described earlier (Z01 HL 01437-02 LTD), the two solvent phases in a rotating coil establishes a hydrodynamic equilibrium in such a way that they are distributed at a given volume ratio in each helical turn on the head side of the coil while any excess of either phase remains at the tail of the coil. This hydrodynamic equilibrium can be efficiently utilized for performing countercurrent chromatography (CCC). When the coil is eluted with one of the phases through the head end, the hydrodynamic equilibrium tends to maintain the original equilibrium volume ratio of the two phases in the coil and thereby a certain volume of the other phase is permanently retained in the coil while the two phases are undergoing vigorous agitation with rotation of the coil. Consequently, the solutes introduced locally at the inlet of the coil are subjected to an efficient partition process between the two phases and chromatographicallly separated according to their partition coefficients in the absence of solid supports.

The present method uses a new type of a coiled column called "multi-layer coil" which consists of multiple layers of the coil as seen in a reel. This column is subjected to a particular type of synchronous planetary motion, i.e., both revolution and rotation of the same the coil being at angular veolocity in the same direction. This produces an extremely high value of the equilibrium volume ratio for the lighter phase ranging from 10 to infinite while the equilibrium volume ratio in other CCC schemes is usually around 1. This high equilibrium volume ratio provides retention of a large volume of the stationary phase in the column against a high flow rate of the mobile phase. Consequently the sizable separations are completed in a short period of time ranging between 2 and 5 hours.

b) Apparatus

The apparatus used in the present studies is essentially the same as the toroidal coil planet centrifuge previously reported (Z01 HL 01437 LTD) except that the following modifications were made.

For the preliminary studies, a large pair of flanges was mounted on the column holder (beta 0.5) of the toroidal coil planet centrifuge to accommodate a multi-layer coil. Although the apparatus produced satisfactory results on separations of DNP amino acid, it had various shortcomings such as difficulty in mounting the column on the holder, difficulty in balancing the apparatus, limitation in space for mounting the column, etc. Improvement was made in a new centrifuge as follows:

1) The column holder was made removable from the rotary frame by loosening a pair of screws. In this way the multi-layer coiled column can be made easily and different types of the column can be tested in the same

apparatus simply by switching the column.

2) The column holder was equipped with a large pair of flanges which provide ample space for the coiled column.

3) The other side of the holder was similarly made removable from the rotary frame and exclusively used as a counterbalance so that balancing of the centrifuge can be done accurately outside of the apparatus.

The multi-layer coiled column was prepared from a single piece of PTFE tubing, typically 1.6 mm i.d. and 130 m long, by winding it tightly onto the coil holder. To prevent dislocation of the column from the holder, each layer of the coil was taped to the flanges by applying a piece of fiberglass adhesive tape across the width of the coil. Each terminal of the column was directly connected to a flow tube of 0.85 mm i.d.

c) Separation Procedure

In each separation the column is first entirely filled with the stationary phase which was preequilibrated with the mobile phase in a separatory funnel in room temperature. The sample solution, prepared by dissolving the sample in the upper and/or lower phase, is introduced through the sample port. Then, the apparatus was run at 800 rpm while the mobile phase was pumped into the column at a given flow rate. The coiled column is always rotated in such a direction that the inside terminal becomes the head and the outside terminal, the tail. The column is eluted through the head end when the mobile phase is the lower phase and vis versa to favor the retention of the stationary phase. The eluate is continuously monitored with an LKB Uvicord S at 280 nm and fractionated into test tubes with an LKB fraction collector.

d) Results

Capability of the apparatus was first demonstrated on separation of a set of dinitrophenyl (DNP) amino acids with a solvent system composed of chloroform, glacial acetic acid and 0.1N hydrochloric acid at a volume ratio of 2:2:1. The separations were performed under various flow rates using both the upper and the lower phases as the mobile phase. The results indicated that the method is capable of separating the samples at a high partition efficiency close to 1000 theoretical plates in 2 to 5 hours. Either aqueous or nonaqueous phase can be used as the mobile phase with similar results. The maximum sample loading tested was 650 mg.

In order to test applicability of various two-phase solvent systems to the method, experiments were conducted to separate various biological samples which include oligopeptides, natural and synthetic polypeptides, indole plant hormones, purines and pyrimidines, etc. The results show that the two-phase solvent systems with a variety of physical properties are well applicable to the present method. A gradient elution was also successfully performed for separation of oligopeptides.

Significance to Biomedical Research and the Program of the Institute:

Separation and purification of biological samples are essential for most of the biomedical studies. CCC is particularly useful for preparative-scale separations because the method eliminates all complications arising from the use of solid supports such as sample loss, denaturation and contamination. The new CCC method described above is capable of yielding a highly efficient preparative separation in a few hours which would take overnight to days of elution with the exisitng CCC devices.

Proposed Course:

1. Application of various columns for large-scale to micro-scale separations.

2. Separation of proteins and other macromolecules.

Publications:

1. Ito, Y.: Efficient Preparative Counter-Current Chromatography with a coil planet centrifuge, J. Chromatogr. 214 (1981) 122-125.

2. Ito, Y., Sandlin, Jesse and Bowers, W. G.: High Speed Preparative Countercurrent Chromatography (CCC) with a Coil Planet Centrifuge, J. Chromatogr., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01446-01 LTD

PERIOD COVERED

October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)

Development of the Non-Synchronous Flow-Through Coil Planet Centrifuge for Separation of Cells and Macromolecules

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Y. Ito	Medical Officer	LTD NHLBI
Others:	G. Bramblett	Biological Aid	LTD NHLBI
	R. Bhatnagar	Biological Aid	LTD NHLBI
	M. Huberman	Medical Officer	LMH NHLBI
	L. Leive	Sr. Investigator	LBP NADDKD

COOPERATING UNITS (if any)

None

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Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 5	PROFESSIONAL: 3	OTHER: 2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The newly built non -synchronous coil planet centrifuge almost completely eliminated noise and vibration which limited the application of the previous model. The apparatus holds a coiled column assembly which is rotated at a freely adjustable rate in a given centrifugal force field. Flow is introduced through the running column without the use of rotating seals. The apparatus is capable of performing efficient countercurrent chromatography (CCC) with polymer phase systems and elutriation of cells with physiological solutions. The versatility of the present scheme has been demonstrated by the partition of E. coli plasmid DNA and Salmonella typhimurium strains and by elutriation of mammalian cells.

1048

Methods Employed and Major Findings:

a) Apparatus:

The improved non-synchronous flow-through coil planet centrifuge has been constructed. The new design completely eliminated the use of the spider gear sets which caused a considerable noise and vibration in the previous model to limit the practical operation. The rotor of the present apparatus consists of two major rotary structures, i.e, frames I and II which are coaxially bridged together with the center piece. Frame I consists of 3 aluminum plates rigidly linked together and holds three rotary elements, the center piece at the center and countershafts I and II, all embedded in ball bearings. Frame II consists of three pairs of arms linked together to rotate around the central shaft. It supports a pair of rotary shafts, one holding coil assembly and the other, serving as a counterbalance.

There are two motors, motors I and II to drive the rotor. Motor I directly drives frame I and, by means of a set of pulleys and gears on countershaft I, rotates the center piece at a speed twice that of the motor in the same direction. Motor II drives frame II through a set of idler pulleys and gears on countershaft II at a desirable rate. Because of a 1:1 ratio pulley coupling between the center piece and the coil holder on frame II, the coil assembly on the holder undergoes a planetary motion in such a way that the rates of rotation and revolution are freely variable by selecting the rates of motors I and II.

The apparatus revolves smoothly up to the maximum rate of 1000 rpm to provide approximately 150 x g on the coil assembly. The rotational speed of the coil assembly can be selected anywhere between 0 to 50 rpm in either direction.

b) Countercurrent chromatography (CCC) with poymer phase systems:

When a water-filled coiled tube is rotated slowly in the gravitational field, any object either heavier or lighter than the water tends to move toward the end of the coil. This end is called the head and the other end, the tail. When such a coil contains two immiscible solvent phases, the rotation soon establishes a hydrodynamic equilibrium where each helical turn is occupied by nearly equal volumes of the two phases and any excess of either phase remains at the tail end of the coil. Under this hydrodynamic equilibrium condition, continuous elution of either phase through the head of the coil permits retention of the other phase in each helical turn while the two phases are constantly mixed by rotation of the coil. Consequently, solutes or particles introduced locally at the head of the coil are subjected to an efficient partition process resulting in chromatographic separation according to their partition coefficients. This countercurrent chromatographic scheme has been successfully applied with the conventional two-phase solvent systems by the synchronous coil planet centrifuge. Because of its freely adjustable rotational rate under a given centrifugal force field, the non-synchronous flow-through coil planet centrifuge further expands the application of the CCC to separation of cells and macromolecules with polymer phase systems which are characterized by extremely low

interfacial tension with a high tendency of emulsification. Preliminary experiments were performed to demonstrate the versatility of the present method which can separate a broad spectrum of samples. For solute separation, plasmid DNA was separated from RNAs and for cell separation Salmonella typhimurium G30 cells were partitioned according to their membrane characteristics.

For plasmid DNA separation, crude preparation of the plasmid pBR 322 was obtained from a lysate of E. coli C600 r^-m^-. The polymer phase system composed of 5% (w/w) dextran 500, 4% (w/w) polyetheylene glycol 6000, and 10 mM sodium phosphate (pH 6.8) was equilibrated in a separatory funnel and separated before use. In each separation, the coiled column was first filled with the stationary lower phase, and the sample solution containing 100 microgram DNA was injected through the sample port located on the flow line between the inlet of the column and the outlet of the pump. Then the mobile upper phase was pumped through the head of the column at 8.5 ml/h, while the apparatus was run at 1000 rpm combined with 5 rpm coil rotation. The results of the experiments show that the plasmid DNA, eluted immediately after the solvent front was separated from the RNAs which were rather evenly partitioned between the mobile and the stationary phases. Although there is some degree of overlap between the two peaks, pooling of fractions containing DNA but not RNA as determined on agarose gel electrophoresis, yielded recoveries of 74% for DNA loaded. Boiling of the sample for 2 minutes followed by quick cooling in a dry ice-ethanol bath, prior to loading, resulted in the separation of closed circular plasmid DNA from denatured linear DNA, of which greater than 90% is retained in the stationary phase. The largest sample loading attempted was 400 micrograms of DNA, with equal resolution obtained.

For partition of cells on polymer phase systems, samples of Salmonella typhimurium differring in cell surface lipopolysaccharide were prepared by growing S. typhimurium strain G30 which lacks UDP glucose-4-epimerase in proteose peptone-beef extract medium, either with galactose or in its absence. In the presence of galactose, this stain makes a normal lipopolysaccharide; in its absence, it makes a short chain terminated at the point of the first galactose in the molecule. Separation of these two bacterial samples was performed with a polymer phase system composed of 6.2% (w/w) dextran 500, 4.4% (w/w) polyethylene glycol 6000, 0.05 M Tris-Cl (ph 7.0), 10 mM potassium phosphate (pH 7.0) and 0.01% sodium azide. In each separation the column was filled with the polymer phase system consisting of about equal amounts of the upper and the lower phases, and galactose (+) and galactose (-) cell mixture suspended in 1 ml of the upper phase was injected through the sample port. Then, the upper mobile phase was pumped through the head of the column at 8.5 ml/h while the apparatus was run at 1000 rpm with 5 rpm coil rotation. The obtained fractions were diluted with distilled water and the elution profile of cells was determined by measuring the turbidity at 280 nm with a Beckman DU spectrophotometer. The results show that cells grown without galactose are mostly partitioned either at the interface or in the lower phase and retained in the column for a long period of time while the cells grown with galactose are mostly distributed in the upper mobile phase and eluted from the column much earlier.

c) Cell elutriation with physiological solution

When a water-filled coiled tube is rotated about its horizontally placed axis, particles present in the coil tend to move toward the head of the coil. With a slow rotational speed, particles of mixed size and density always stay together at the bottom of the coil and move toward the head at a rate of one helical turn per one rotation of the coil without separation. When the rotational rate is increased, the small and/or lighter particles are retarded in their movement toward the head resulting in chromatographic separation of the particles according to their sedimentation rate. In this situation continuous flow can be introduced through the coil to fractionate separated particles. Separation of cells can be performed similarly with the non-synchronous flow-through coil planet centrifuge under a centrifuged force field. Capability of the apparatus for cell elutriation is demonstrated on separation of human and sheep erythrocytes with a buffered isotonic saline solution. In each separation the column was filled with the saline solution and the sample cell mixture was introduced at the inlet of the coil. Then, the apparatus was rotated at 1000 rpm combined with a slow coil rotation. The elution was performed through either head or tail of the coil. The head-tail elution produced elution profile of cells quite similar to the size distribution curve of the original sample obtained by the Coulter Analyzer. The tail-head elution separated cells in such a way that a broad peak of small sheep cells followed by a sharp peak of large human cells. The method was also successfully applied to separation of rat liver cells prepared by means of intravenous collagenase infusion through the portal vein.

Significance to Biomedical Research and the Program of the Institute:

Separation of macromolecules and cells is one of the most important steps in many biomedical studies. The present scheme introduces gentle separation procedures which can preserve biochemical and physiological nature of biological samples. Partition of cells and macromolecules may be further extended to affinity chromatography by the use of ligand-bound polymers which are now under intensive development in many research laboratories.

Proposed Course:

Application of the method to various biological samples.

Publications:

1. Ito, Y.: Minireview: Countercurrent Chromatography, J. Biochem. Biophys. Met. 5 (1981) 105-129.

2. Ito, Y.: Countercurrent Chromatography: Principle and Application, Protein, Nucleic Acid and Enzyme 26, No. 8 (1981) 1020-1046.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01447-01 LTD

PERIOD COVERED
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)
Physiochemical Instrumentation in Biochemical/Biomedical Research

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: G. W. Liesegang Sr. Staff Fellow LTD NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1. The development of a (mode-locked) picosecond flash photolysis instrument has been completed and tested. The spectrometer has a sensitivity of ± 0.01 optical density units and operates at four wavelengths 1064, 532, 355, and 266 nanometers with energies of 60, 10, 1, and 2 millijoules respectively. A dye laser attachment has been constructed so as to improve the tunable excitation range.

2. Design and construction of a nitrogen-laser photolysis apparatus in the subnanosecond range has been started. This instrument overcomes many of the problems normally enocuntered in conventional photolysis instrumentation and provides the biochemist with a versatile flash photolysis instrument.

3. A fluoresence lifetime spectrometer employing a pulsed tunable UV laser has been designed and partially constructed. Intrinsic fluorescence lifetime and energy transfer measurements on proteins are plagued by the necessity of deconvoluting the instrumental response from the decay curves. This new instrument has a response time of less than 200 picoseconds and will permit a direct measurement of intrinsic fluorescence in proteins.

1052

Objectives:

Development and application of physiochemical techniques and instrumentation to the biochemical and biomedical sciences.

Methods:

The instruments and techniques developed under this project are unique systems designed specifically for biochemical investigations and thus contain extensive modifications of some commercially available equipment as well as devices designed and built in our laboratory machine shop.

Major Findings:

1. Flash photolysis has proven a valuable instrumental technique in unraveling the photobiology of such processes as photosynthesis and visual excitation. Extension of this technique into the nano-subnanosecond time regime will allow detection of rapidly formed photo products and their subsequent reactive pathways. Such information is useful not only in detailing mechanism but also in detecting the primary steps involved in UV irradiation of biological systems.

The picosecond flash photolysis apparatus developed in our laboratory has been thoroughly tested over this last year. The system operates at four wavelengths: 1064, 532, 355, and 266 nm with energies of 60, 10, 1, and 2 millijoules respectively. Operation in a single beam pump-probe configuration results in a sensitivity of ± 0.1 ΔOD units. However it was observed that for most biochemical systems an increase in sensitivity to at least ± 0.02 ΔOD units was required. Through development of a double-beam configuration utilizing fiber optics a ± 0.02 ΔOD sensitivity was achieved. To further improve the signal-to-noise ratio and minimize intensity fluctuations in the laser source we have successfully operated the laser at a repetition rate of six pulses per minute, (this being a factor of ten over existing systems) thus allowing signal averaging of the spectra. In this configuration optical density changes of ± 0.01 or better were routinely obtained as well as a significant reduction in the time required to obtain a complete photolysis spectrum. This reduction in experimental time is critical for studies on unstable preparations.

A short cavity dye laser has also been constructed so as to expand the tunability of the excitation wavelength from their discrete values to a broadly tunable range covering 265 to 700 nm. A paper is now in preparation describing this system.

In conjunction with this project we have completed a thorough investigation of the vidicon as a spectroscopic detector under both continuous and pulsed illumination. The results of this work have been used by numerous NIH investigators (e.g. Dr. R. Balaban NHLBI/LKE; Dr. I. Levin NIADDKD/LCP) as well as our consultation to several outside organizations (eg. Dr. Olin Jarrett, NASA - Langley Research Center). Finally Princeton Applied Research Corporation is including some of our techniques into their OMA2 system.

2. The single shot nature of the above technique requires a high energy per pulse which has proven a disadvantage since it introduces unwanted effects such as stimulated emission, excited state annihilation, and/or sample decomposition. Other problems such as poor laser stability, low repetition rate and consequently the high powers, and extreme experimental complexity have contributed to conflicting and erroneous experimental results. In response to this problem I have designed and begun construction on a new instrument which will overcome some, if not all, of the above problems. This instrument will provide the experimenter with a simple and reliable subnanosecond flash photolysis instrument.

The instrument contains many of the components of the older spectrometer but employs a nitrogen pumped dye laser which enables: i) fast temporal pulse excitation (less than 60 picoseconds) ii) high repetition rate (100 Hertz) thus allowing both extensive signal averaging of the spectrum and lower single peak pulse power, iii) excellent pulse selection, and iv) wide spectral tunability (357-700 nm; 245-357 nm frequency doubled).

In preliminary experiments this system is proving much more reliable and sensitive thereby making it a viable research tool to the biochemist. For example, in photolysis experiments changes in optical density of 10^{-3} are easily detectable with minimum signal averaging. Experiments are now in progress to determine the minimum ΔOD obtainable.

3. In proteins with multiple tryptophans excited at 280 nm one can analyse the emission lifetimes and detail which tryptophan is being quenched by energy transfer to a binding chromophore. For delineating intramolecular distances the emission lifetime must be precisely separated for each tryptophan. Analysis of the tryptophan lifetime by conventional lifetime instruments such as the Ortec 9200 fluorescence spectrometer requires deconvolution of the 2-3 nanosecond lamp pulse and therefore adds considerable uncertainty to the accuracy of the multiple decaying lifetimes.

Given the short pulse width, UV output, and high photon flux of the nitrogen laser it would appear to be an ideal light source in developing a fluorescence lifetime instrument which would yield directly lifetimes down to 150 picoseconds. In this vein the design and construction of a nitrogen laser pumped dye laser fluorescence lifetime spectrometer is in progress. A high speed (100 picosecond) phototube is being employed as the detector together with a boxcar averager with a 75 picosecond dead time. The overall instrumental response time and sensitivity are presently being investigated.

This instrument when completed will be used in collaboration with several NIH investigators to detail the molecular mechanism of protein binding.

Significance to Biomedical Research and the Program of the Institute:

The use of physiochemical instrumentation is of fundamental importance in biomedical research. Notably the development of spectroscopic

instrumentation for observing structural changes within a protein macromolecule are important in understanding biochemical processes on a molecular basis.

Proposed Course of Research:

1. We plan to complete the project on the picosecond laser system and prepare our results for publication. Additional collaboration with other laboratories, using this system, has already been started and will be extended. For instance we are investigating the role of mode-locked (picosecond) lasers and its associated instrumentation to opthamological surgery. This project is a joint collaboration with Dr. Douglas Gaasterland, NEI/CB.

2. The picosecond fluorescence lifetime system will be completed and research projects carried out in collaboration with Dr. Harold Edelhoch (NIAMDD/LCE), Dr. Randall Kincaid (NHLBI/CM), and Professor Enrico Bucci (University of Maryland Medical School, Dept. of Biological Chemistry).

3. A program in collaboration with Dr. Robert Balaban (NHLBI/LKE) will be started to develop physical-chemical instrumentation which maximizes fluorescence emission while minimizing photobleaching, in the study of renal cellular transport mechanisms.

4. The development of a vidicon based room temperature phosphorescence spectrometer for the determination of rotational depolarization of extrinsic and intrinsic phosphorescent probes.

5. Investigation into the applicability of Raman spectroscopic techniques to calcium measurement in cells and as a method to determine the chemical and structural composition of systems in biological organisms.

Publications:

Liesegang, G. W. and Smith, P. D.: Improving vidicon linearity in the pulsed illumination mode. Appl. Optics 20: 2604-2605, (1981).

Liesegang, G. W. and Smith, P.D.: Vidicon characteristics under continuous and pulsed illumination. Applied Optics 21: 1437-1444, (1982).

Cascio, H., Smith, P. D., and Liesegang, G. W.: A switching circuit to improve vidicon (OMA2) linearity. Rev. Sci. Instru. , July (1982).

Liesegang, G. W. and Shapiro, S. L.: Subnanosecond fluorescence quenching of Auramine O. In Picosecond Phenomena III. Springer-Verlag, 1982.

Liesegang, G. W. and Smith, P.D.: Workshop on vidicons as spectroscopic detectors. Biophysical Discussions: Protein-lipid interactions in membranes Biophys. J. 37, (1982).

ANNUAL REPORT OF THE
LABORATORY OF BIOCHEMICAL GENETICS
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1981 through September 30, 1982

Monoclonal antibodies were obtained that affect transsynaptic communication, inhibit intercellular adhesion, recognize antigens associated with the synaptic layers of retina, or identify certain types of cells in retina. Five antibodies were obtained that bind to antigens that are restricted to the synaptic layers of retina; three of the antigens are restricted to the outer synaptic layer, one to the inner synaptic layer, and one to both the inner and outer synaptic layers of retina. The latter antibody also markedly inhibits intercellular adhesion of retina cells. Two antibodies are specific for retina ganglion neurons, seven for rods and cones, and seven for Muller cells. Other antibodies recognize various sets of neurons; such as horizontal, bipolar, and amacrine neurons of the inner nuclear layer of retina.

Monoclonal antibodies also were obtained which recognize neuroblastoma-hybrid cell antigens. Five antibodies were found that increase the frequency of miniature endplate potentials of myotubes innervated by the hybrid cells; whereas, six antibodies markedly decrease the frequency of miniature endplate potentials. The antibodies that affect rates of acetylcholine secretion from hybrid cells do not alter the cell membrane potential.

In previous studies we showed that a protein in avian retina, termed Top, is distributed in a large dorsal-ventral gradient. The protein thus defines a dorsal-ventral axis of the retina and identifies the relative positions of cells in retina with respect to this axis. During the past year the protein was purified extensively; the apparent M_r of the protein is 47,000 and the isoelectric point is approximately 4.1. A monoclonal antibody directed against Top in retina also recognizes a protein in chick cerebral cortex and thalamus. The protein in brain was purified and characterized; the M_r and isoelectric point of the protein from brain were the same as that found for Top from retina. At this stage, we know relatively little about the regulatory mechanism which relates the number of molecules of the protein detected per cell with the relative positions of the cell in the retina, except that cells dissociated from retina that were cultured in vitro continue to express the amount of the protein that would be expected based on the original position of the cell in the intact retina. Further studies on both the structure of the protein and the mechanism regulating the expression of the protein are in progress.

The species of transmitter synthesized by a neuron determines, at least in part, the type of neuron and the kinds of synaptic connections that are formed. A project was initiated to serve as a foundation for future studies on mechanisms that regulate the expression of choline acetyltransferase genes. Choline acetyltransferase from rat brain was purified approximately 100,000-fold and mice then were injected with purified enzyme preparations. Spleen cells from the immunized mice were fused with P3X63 Ag8 myeloma cells; four of the hybridoma cell lines that were obtained were found to synthesize antibodies directed against choline acetyltransferase. The current objective is to use the antibodies for the purification of mRNA coding for choline acetyltransferase in order to clone the corresponding molecules of cDNA by recombinant DNA methods.

Sixty five monoclonal antibodies were obtained which bind specifically to membrane preparations from NG108-15 neuroblastoma-glioma cells. Six antibodies were found which bind to a greater extent to membranes from differentiated NG108-15 cells than to membranes from undifferentiated NG108-15 cells. Two-dimensional gel electrophoresis also revealed several proteins which are more abundant in membranes from differentiated cells, compared to membranes from undifferentiated cells.

We previously showed that the acquisition of functional voltage-sensitive calcium channels by NG108-15 cells enables the cells to form synapses with striated muscle cells. A 1,4-dihydropyridine analog, [^{3}H]-nitrendipine, which reportedly binds specifically to voltage-sensitive calcium channels, was used as a probe for voltage-sensitive calcium channels of NG108-15 cells. [^{3}H]-Nitrendipine was shown to bind specifically to membranes prepared from differentiated NG108-15 cells; the dissociation constant, determined by Scatchard analysis, was approximately 2×10^{-10} M. The number of specific binding sites for [^{3}H]-nitrendipine per average cell was shown to increase 10-fold, from 1,600 to 16,000, when cells were treated for 3 or more days with PGE_1 which activates adenylate cyclase and elevates cellular cAMP levels. These results suggest that prolonged elevation of cellular cAMP levels results in an increase in the number of voltage-sensitive calcium channels per cell and that the ability of the cells to form synapses is regulated by the rate of synthesis and/or turnover of voltage-sensitive calcium channels.

NG108-15 cells contain large, dense-core vesicles and small, clear vesicles. Cells with endogenously synthesized [^{3}H]-acetylcholine were lysed and the vesicles that were released were separated from cell membranes and then fractionated by sucrose density centrifugation. Two well-separated peaks of acetylcholine were found, which comprised greater than 90% of the intracellular [^{3}H]-acetylcholine found. Similar results were obtained with a mutant cell line which lacks large dense-core vesicles, but has large vesicles with amorphous electron-lucent cores. These results suggest that acetylcholine in NG108-15 cells is stored in large dense-core vesicles and small clear vesicles and that the clonally inherited change in vesicle morphology in the varient cell line does not affect the ability of the vesicles to store acetylcholine.

A protein was obtained from bovine brain which markedly stimulates neurite outgrowth by cultured neurons dissociated from chick embryo cerebral cortex. The protein was purified approximately 200-fold. The native protein is a dimer with an apparent M_r of 75,000 which on reduction dissociates into subunits with an M_r of 37,000. The protein, estimated to be $>$ 90% pure, induces neurite outgrowth at a concentration of approximately 1 nM. A rabbit antiserum to the neurite extension factor, termed NEF, was obtained which blocks the activity of the protein in stimulating neurite extension. Neurite extension protein was detected by immunohistochemical methods in neurons in some, but not all, regions of adult rat brain. These results suggest that NEF is required for neurite extension by some neurons in the central nervous system.

NG108-15 cells release a protein into the medium which induces the formation of clusters of nicotinic acetylcholine receptors on myotube plasma membranes. Laminin, a myotube basement membrane glycoprotein, was shown to potentiate the aggregation of the acetylcholine receptors. Basement membrane proteins of myotubes such as laminin, collagen types IV and V, and heparin

sulfate proteoglycan were detected on the surface of muscle fibers at early stages of receptor aggregation in vivo. The basement membrane proteins associated with acetylcholine receptor aggregates were relatively resistant to extraction by detergent. Exposure of myotubes to brain extracts resulted in marked aggregation of acetylcholine receptors on myotubes and the appearance of cytoskeletal specializations under myotube plasma membranes. These results suggest that the binding of laminin to the muscle cell surface and the formation of cytoskeletal structures beneath the cell membrane may promote the formation of nicotinic acetylcholine receptor aggregates and/or stabilize the receptor aggregates.

Polyadenylated mRNA was purified from adrenal medulla and brain and was used to direct the cell-free synthesis of preproenkephalin, a precursor of opioid enkephalin pentapeptides. The apparent M_r of preproenkephalin was found to be 30,000. The bovine precursor was shown to contain the amino acid sequences of both methionine- and leucine-enkephalin. Preproenkephalin was converted to a smaller protein, termed proenkephalin, with an apparent M_r of 28,000. Monoclonal antibodies directed against Met-enkephalin and Met-enkephalin [ArgPhe] were obtained. A rat brain cDNA library was prepared by recombinant DNA techniques which will be screened for rat preproenkephalin clones and other clones of interest. cDNA hybridization probes then will be used to study the regulation of enkephalin gene expression. In a related project, corticotropin-releasing factor was shown to stimulate the secretion of beta-endorphin and corticotropin from clonal AtT-20 mouse pituitary tumor cells and to increase the activity of adenylate cyclase of the cells.

Histidyl-proline diketopiperazine, after injection into rats, was cleared from the circulation with biphasic kinetics ($t_{1/2}$ = 1.25 and 33 min). Unmetabolized histidyl-proline diketopiperazine appeared rapidly in the urine. The longer half-time of clearance of the peptide of 33 min suggested a tissue reservoir of the peptide. Histidyl-proline diketopiperazine was found to accumulate in adrenal, liver, and kidney. Therefore, specific binding of the diketopiperazine to membrane preparations was examined. Such binding was observed in membrane preparations derived from adrenal and liver.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00009-08 LBG

PERIOD COVERED
October 1, 1981 - September 30, 1982

TITLE OF PROJECT (80 characters or less)

Cell Recognition and Synapse Formation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Marshall Nirenberg	Chief, LBG	LBG, NHLBI
OTHER:	Howard Burrows	Guest Worker	LBG, NHLBI
	Hiroyuki Fukai	Visiting Fellow	LBG, NHLBI
	Gerald Grunwald	Guest Worker	LBG, NHLBI
	Karl Krueger	Guest Worker	LBG, NHLBI
	Joseph Moskal	Staff Fellow	LBG, NHLBI
	William Strauss	Staff Fellow	LBG, NHLBI
	David Trisler	Staff Fellow	LBG, NHLBI
	Ilan Spector	Visiting Associate	LBG, NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemical Genetics

SECTION
Section of Molecular Biology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
12	9	3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Monoclonal antibodies were obtained that affect transsynaptic communication, inhibit intercellular adhesion, recognize antigens associated with the synaptic layers of retina, or are expressed by specific cell types in retina such as ganglion neurons, rods and cones, or Muller cells. Five antibodies were obtained that increase the rate of acetylcholine secretion from neuroblastoma-hybrid cells, whereas six antibodies decrease the rate. Another antibody recognizes a 47,000 M_r protein isolated from retina cell membranes, which is distributed in a dorsal-ventral topographic gradient in avian retina. A similar protein also was purified from chick cerebral cortex and thalamus. In addition, monoclonal antibodies were obtained to choline acetyltransferase from rat brain. Evidence was obtained which suggests that acetylcholine of NG108-15 neuroblastoma-glioma hybrid cells is stored in large dense-core vesicles and small clear vesicles and in abnormal large vesicles which lack dense-cores which are produced in a varient cell line.

1059

Objectives:

To identify and characterize molecules involved in synapse formation and function.

Major Findings:

Monoclonal antibodies were obtained that affect transsynaptic communication, inhibit intercellular adhesion, recognize antigens associated with the synaptic layers of retina, or identify certain types of cells in retina. Five antibodies were obtained that bind to antigens that are restricted to the synaptic layers of retina; three of the antigens are restricted to the outer synaptic layer, one to the inner synaptic layer, and one to both the inner and outer synaptic layers of retina. The latter antibody also markedly inhibits intercellular adhesion of retina cells. Two antibodies are specific for retina ganglion neurons, seven for rods and cones, and seven for Muller cells. Other antibodies recognize various sets of neurons; such as horizontal, bipolar, and amacrine neurons of the inner nuclear layer of retina.

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Significance to Biomedical Research:

New information was obtained concerning synaptogenesis and synaptic function.

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12. Spector, Ilan, Sochet, Nava R., Kashman, Yoel and Groweiss, Amiram: Latrunculins: Novel Marine Toxins That Disrupt Microfilament Organization In Cells. Science, In Press (1982).

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00017-07 LBG

PERIOD COVERED
October 1, 1981 - September 30, 1982

TITLE OF PROJECT (80 characters or less)

Acetylcholine Receptors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Mathew P. Daniels	Research Biologist	LBG NHLBI
OTHERS:	Hans Bauer	Visiting Scientist	LDN NICHD
	Peter Sonderegger	Guest Worker	LDN NICHD
	Zvi Vogel	Guest Worker	LBG NHLBI
	Anthony Olek	Guest Worker	LBG NHLBI

COOPERATING UNITS (if any)

Laboratory of Developmental Neurobiology, NICHD

LAB/BRANCH
Laboratory of Biochemical Genetics

SECTION
Section on Molecular Biology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.0	2.5	2.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Our aim is to study the organization of neurotransmitter receptors on nerve and muscle cells in relationship to the development and function of synapses. We have used α-bungarotoxin as a specific probe for the visualization and quantitation of nicotinic acetylcholine receptor sites. Our recent work has focused upon the factors, extrinsic and intrinsic to the developing skeletal muscle fiber, which regulate the distribution of nicotinic acetylcholine receptors. We have now provided further evidence for a role of a basement membrane glycoprotein, laminin, in modulating the receptor aggregation induced by neuronal factors. This modulation appears to depend on the binding of laminin to the muscle cell surface. We have also shown that receptor aggregation can be rapidly induced by neuronal factors, with correlated changes in cytoskeletal structures beneath the cell membrane.

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Project Description:

Objectives:

Our aim has been to study the organization of neurotransmitter receptors on nerve and muscle cells in relationship to the development and function of synapses. Our recent work has focused on factors, extrinsic and intrinsic to the developing muscle fiber, which regulate the distribution of nicotinic acetylcholine (ACh) receptors. In particular, we have been investigating the induction of ACh receptor aggregation on muscle cells in culture, by soluble macromolecules from neurons, as a model for the aggregation of ACh receptors which occurs at the developing neuromuscular synapse.

Methods:

We have stained skeletal myotubes grown in monolayer culture with rhodamine-labeled -bungarotoxin (BT) in order to visualize ACh receptor sites with the fluorescence microscope. Sequential observations of ACh receptor distribution were made by use of a video image intensification system.

The distribution of basement membrane proteins in the cultures, and in sections of muscle tissue, was determined by indirect immunofluorescence, using antisera against purified proteins.

Alpha-bungarotoxin coupled to horseradish peroxidase was used to examine the distribution of ACh receptors on myotube surfaces at the electron microscopic level.

ACh receptor aggregating material was prepared from culture medium conditioned by clonal nerve cell lines or from extracts of fetal pig brain.

Major Findings:

1. The role of basement membrane proteins in the organization of acetylcholine receptors.

Laminin was consistently found to be associated with regions of high ACh receptor density (ACh receptor aggregates) which had formed de novo in the presence of neuronal macromolecules, as well as with pre-existing aggregates, as shown before. The association of laminin and other basement membrane proteins with receptor aggregates was resistant to detergent extraction, suggesting a stable interaction between extracellular matrix and aggregated ACh receptors. Basement membrane proteins (laminin, collagen types IV and V and heparan sulfate proteoglycan) were detected on the muscle fiber surface at the earliest stages of receptor aggregation at the synapse, in vivo.

Results of kinetic and immunofluorescence experiments suggested that laminin binds to the surface of cultured myotubes and, once bound, may stabilize the neuronal macromolecules which induce ACh receptor aggregation, or stabilize the aggregates themselves, thus causing the previously observed enhancement of receptor aggregation.

2. Rapid changes in ACh receptor distribution:

After exposure of myotubes to embryonic brain extract for thirty minues to one hour, marked aggregation of ACh receptors occurred within approximately three hours. The aggregation process is light-sensitive, but can be observed directly by use of low illumination and video image intensification. Rapid aggregation of receptors on the myotube surface is correlated with the appearance of cytoskeletal specializations under the plasma membrane, similar to those previously found in pre-existing receptor aggregates.

Significance to Biomedical Research:

An understanding of the control mechanisms involved in the organization of neurotransmitter receptors at the developing synapse is of clear importance in anyt attempt to understand the role of neurotransmitters and their receptors in the function and development of the nervous system. Our studies on the interactions between neuronal factors, extracellular matrix components, and cytoskeletal structures may lead to a better understanding of the mechanisms whereby neurons control or modulate the distribution of receptors on muscle fibers and on other neurons, during synapse development and after.

Proposed Course:

1. The detailed relationship between basement membrane protein distribution and ACh receptor aggregation in myotubes developing in vivo will be studied, using light and electronmicroscopic immunohistochemistry.

2. Ultrastructural changes correlated with rapid and long term changes in ACh receptor organization will be examined by electron microscopy following direct observation of these changes in living myotubes.

Publications:

Prives, J., A. B. Fulton, S. Penman, M.P. Daniels, and C. N. Christian. 1982. Interaction of the cytoskeletal framework with acetylcholine receptor on the surface of embryonic muscle cells in culture. J. Cell Biol. 92: 231-236.

Schaffner, A. E. and M. P. Daniels, 1982. Conditioned medium from cultures of embryonic neurons contains a high molecular weight factor which induces acetylcholine receptor aggregation on cultured myotubes. J. Neurosci. 2: 623-632.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00018-05 LBG

PERIOD COVERED
October 1, 1981 - September 30, 1982

TITLE OF PROJECT (80 characters or less)

Regulation of the biosynthesis and secretion of opioid peptides

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Steven L. Sabol	Medical Officer (Research)	LBG, NHLBI
Others:	Satyaprabha Dandekar	Visiting Fellow	LBG, NHLBI
	Chi-Ming Liang	Senior Staff Fellow	LBG, NHLBI

COOPERATING UNITS (if any)
Vivian Hook, Seymour Heisler, and Julius Axelrod (Lab. Clinical Science, NIMH)

LAB/BRANCH
Laboratory of Biochemical Genetics

SECTION
Section on Molecular Biology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.3	3.0	0.3 (Summer student)

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The biosynthesis of the opioid pentapeptides enkephalins is under investigation. The primary enkephalin precursor, called preproenkephalin, was synthesized and studied by cell-free translation of messenger RNA from brain and adrenal medulla. The molecular weight of bovine, rat, and guinea pig preproenkephalin is 30,000. The bovine precursor was shown to contain both methionine- and leucine-enkephalin. Preproenkephalin and its biosynthetic intermediates were detected and quantitated by immunoprecipitation with anti-Met-enkephalin[ArgPhe] serum, followed by gel electrophoresis. Preproenkephalin was shown to be cotranslationally converted by microsomes to an apparently unglycosylated protein of M_r 28,000, called proenkephalin. Bovine preproenkephalin mRNA possesses 1450 + 200 nucleotides. Monoclonal antibodies have been prepared against Met-enkephalin and Met-enkephalin[ArgPhe]. A rat brain cDNA library has been prepared by recombinant DNA techniques for isolation of rat preproenkephalin clones and other clones of interest. The regulation of enkephalin gene expression is under investigation. In a related project, corticotropin-releasing factor (CRF) was shown to stimulate the release of beta-endorphin and corticotropin from clonal AtT-20 mouse pituitary tumor cells, as well as to stimulate adenylate cyclase of these cells.

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Project Description:

Objectives:

The pentapeptides methionine-enkephalin and leucine-enkephalin, and the larger peptide beta-endorphin, are endogenous opiate-receptor ligands that may function as neuromodulators. Other laboratories have characterized enkephalin-containing peptides from adrenal medulla, which are fragments of a larger, not yet isolated, precursor called proenkephalin. We have adopted the following objectives: (A) the synthesis and characterization of the unmodified enkephalin precursor (gene product) and characterization of enkephalin precursor mRNA, (B) cloning and sequencing of the enkephalin precursor cDNA, (C) studies to determine how enkephalin biosynthesis is regulated, and (D) the generation of monoclonal antibodies that will recognize various regions of the enkephalin precursor. In a related aspect (part E) a clonal ACTH/beta-endorphin secreting pituitary tumor cell line was studied to determine whether it responds to the recently described corticotropin-releasing hormone.

Methods Employed:

Discussed in major findings below.

Major findings:

A. Enkephalin precursor translation

Polyadenylated mRNA was purified from bovine adrenal medulla, bovine and guinea pig striatum, and rat whole brain (minus cerebellum). mRNA was translated in the rabbit reticulocyte and wheat germ cell-free translation systems, and the synthesis of enkephalin precursor was assayed by two methods. In the first (older) method, cell-free translation products labeled with [^{35}S]methionine are treated with trypsin and carboxypeptidase B to liberate [^{35}S]Met-enkephalin, which is then isolated by immunoprecipitation with anti-Met-enkephalin antibodies and subsequent HPLC fractionation. In the second (newer) method, protein(s) containing the Met-enkephalin sequence are directly immunoprecipitated by affinity-purified antibodies raised against Met-enkephalin-Arg^6-Phe^7. Immunoprecipitated ^{35}S-labeled proteins are analyzed by SDS-polyacrylamide gel electrophoresis and subsequent fluorography. Met-enkephalin-containing proteins are identified by the criterion of immunocompetition by unlabeled Met-enkephalin-Arg^6-Phe^7.

Using the first method we demonstrated that cell-free translation of mRNA from bovine adrenal medulla and striatum results in the synthesis of an M_r 31,000 $\pm$ 1000 protein that contains [^{35}S]Met-enkephalin (up to 0.43 fmol synthesized per microgram added mRNA). Approximately 0.017% of the [^{35}S]methionine incorporated into protein was recovered in [^{35}S]Met-enkephalin sequences. Both [^{3}H[Met-enkephalin and [^{3}H]Leu-enkephalin were recovered in a molar ratio of 4 $\pm$ 1 to 1 from proteins of M_r 31,000 $\pm$ 3000 obtained by translation of striatum mRNA in the presence of [^{3}H]amino acids. [^{35}S]Met-enkephalin was obtained from

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proteins encoded by mRNA from bovine striatum and adrenal medulla, tissues rich in enkephalin, but not adrenal cortex or liver, tissues having little or no enkephalin. Optimal conditions of enkephalin precursor synthesis were determined; the most important finding was that subsaturating concentrations of mRNA are necessary for optimal precursor synthesis.

To determine the size of enkephalin precursor mRNA, total mRNA was fractionated on methylmercury-agarose gels, and each fraction was translated and assayed for enkephalin synthesis by the first method above. The major enkephalin precursor mRNA of bovine adrenal medulla and striatum was shown to have a length of 1450 $\pm$ 200 nucleotides. A larger species (4750 nucleotides) was sometimes detected in small amounts.

To immunoprecipitate the intact enkephalin precursor according to the second method, antibodies recognizing this precursor, not previously available, were required. An antiserum (RB13) raised by us against the C-terminal heptapeptide of the precursor, Met-enkephalin-Arg^6-Phe^7 was found to recognize larger enkephalin-containing precursors and intermediates. In radioimmunoassay with [^{125}I] Met-enkephalin-Arg-Phe, the RB13 antiserum was found to be directed to the sequence Tyr-Gly-Gly-Phe-Met-X, where X is preferably Arg. The antiserum also reacts with proteins of bovine adrenal medulla extracts having apparent molecular weights of 34,000, 18,000, 11,000, and less than 10,000. The M_r 34,000 protein has the same size as proenkephalin.

Affinity-purified RB13 antibodies immunoprecipitated a putative enkephalin precursor of M_r 30,000 $\pm$ 500 from cell-free translation products encoded by mRNA from bovine adrenal medulla, bovine striatum, guinea pig striatum, or rat brain. Immunoprecipitation of the M_r 30,000 protein was blocked by unlabeled Met-enkephalin-Arg^6-Phe^7. Little or none of this protein was encoded by adrenal cortex mRNA or liver mRNA. To confirm that the bovine M_r 30,000 protein actually contains the Met-enkephalin sequence, the ^{35}S-labeled protein was immunoprecipitated, then treated with trypsin and carboxypeptidase B, and liberated enkephalin residues were then immunoprecipitated and analyzed by HPLC. As predicted, [^{35}S] Met-enkephalin was recovered when the first immunoprecipitation was done in absence but not the presence of excess Met-enkephalin-Arg-Phe.

Addition of dog pancreas microsomes to the cell-free translation systems programmed with adrenal medulla or striatum mRNA resulted in the co-translational conversion of the immunoprecipitated M_r 30,000 protein to a new immunoprecipitated M_r 28,000 protein. We propose that the primary enkephalin precursor (called preproenkephalin according to standard nomenclature) contains a signal peptide that is removed by microsomal signal peptidase to yield the smaller precursor (called proenkephalin). The M_r 28,000 precursor is probably not core-glycosylated, since it does not bind to Concanavalin A-Sepharose.

B. Cloning of enkephalin precursor cDNA

During the past year, attempts have been made by Dr. Dandekar to clone bovine striatum enkephalin precursor cDNA in order to obtain hybridization probes to study enkephalin mRNA regulation and to sequence striatum precursor cDNA.

Cloning was carried out using the M13mp7 phage vector, and recombinant clones were screened for enkephalin mRNA inserts using several methods, including hybridization with a pentadecadeoxynucleotide probe pool complementary to the Met-enkephalin mRNA sequences. However, none of the clones contained enkephalin mRNA. We have nevertheless continued the recombinant DNA work using improved methods and the PBR322 plasmid vector. We have prepared a cDNA library containing clones of cDNAs obtained from the entire rat brain mRNA pool. The library will be used initially to screen for rat preproenkephalin clones using the pentadecadeoxynucleotide hybridization probe. We have shown that this probe hybridizes with bovine striatum mRNA of 1450 nucleotides in length.

C. Regulation of enkephalin biosynthesis

In the first attempt to use the cell-free translation assay to study regulation of enkephalin biosynthesis, we sought to determine whether morphine dependence is associated with a change in preproenkephalin mRNA levels. Groups of rats were addicted for 14 days to high doses of morphine in a slow-release preparation. mRNA was purified from the brains of addicted and sham-addicted rats. The mRNA from addicted animals had similar or slightly increased amounts of preproenkephalin mRNA activity compared to mRNA from the sham-addicted animals.

D. Monoclonal antibodies

The monoclonal antibody (hybridoma) technique was set up in our group by Dr. Liang to obtain specific antibodies to various regions of the enkephalin precursor that would be useful in biosynthetic studies as well as processing studies and immunocytochemistry. Two improvements in the methods already in use at the LBG were made. First, it was found that the yield of hybridomas of spleen cells with non-producing myelomas was improved by the use of PEG-3350 instead of PEG-1000 as the fusion agent. Secondly, it was found that incubation of spleen cells from an immunized mouse with antigen in vitro for 4 days resulted in an improved percentage of hybridomas secreting antibody against the desired antigen, because of selective proliferation of stimulated lymphocytes in culture.
Groups of mice were immunized with Met-enkephalin-hemocyanin, Met-enkephalin-Arg^6-Phe^7-hemocyanin, or peptide E-hemocyanin (peptide E is a fragment of proenkephalin). For the first antigen, 115 hybridomas were obtained and 40 out of 60 surviving clones were positive for binding to antigen-mouse albumin coated polyvinyl plates. Twenty positive clones were recloned, and 6 stable positive hybrids were maintained. For the second antigen, 487 hybridomas were screened, and 290 were positive in binding to antigen-albumin coated plates. The most positive 27 were recloned, but only 4 stable positive clones were finally obtained. Attempts to characterize the antibodies have been problematic, because they apparently do not bind $[^{125}I]$Met-enkephalin or $[^{125}I]$Met-enkephalin-Arg-Phe in solution and bind $[^{3}H]$Met-enkephalin weakly.

E. Mechanism of action of corticotropin-releasing factor (CRF) action

The structure of hypothalamic CRF was reported by Vale's group in September 1981. Synthetic CRF was tested for the ability to stimulate ACTH and

beta-endorphin secretion from clonal AtT-20/D16-16 mouse pituitary tumor cells, previously studied in this lab. Release of both hormones was stimulated maximally about 5-fold. The half-maximally effective concentrations were 4 nM CRF, 10 nM CRF-Met-sulfoxide, greater than 10,000 nM CRF-free acid, and 0.5-2 nM sauvagine, a frog-skin peptide. Preliminary results indicated that CRF may also stimulate the biosynthesis of the ACTH/beta-endorphin precursor. The stimulation of ACTH release was blocked by preincubation of the cells with 10 nM dexamethasone for 6-18 hr.

Exposure of AtT-20/D16-16 cells to CRF for 15 min. resulted in up to 17-fold elevation of intracellular cAMP levels. The half-maximal effect was at 40 nM CRF. In homogenates of these cells, CRF also stimulated adenylate cyclase activity, but higher concentrations of CRF were required (half-maximal effect at 400 nM), possibly due to proteases in the homogenate. These results suggest that the biological effect of CRF may be mediated by activation of adenylate cyclase.

Significance to biomedical research:

A. Our group is the only one so far to have successfully used cell-free translation to study enkephalin biosynthesis and to determine the size of the precursor, the similarities between adrenal medullary and brain preproenkephalin, and the conversion of preproenkephalin to proenkephalin. Our reported size of the precursor (M_r 30,000) was confirmed by sequencing of bovine mRNA by Nakanishi's group, who deduced a size of M_r 29,786. This information provides very important basic knowledge as well as methodology for further studies on the role of the enkephalinergic neuronal/endocrine systems in pain perception, stress, and behavior.

B. Cloning of the rat enkephalin precursor cDNA will provide hybridization probes to quantitate the enkephalin precursor mRNA levels in regulatory studies involving rats. Although bovine and human preproenkephalin clones have been produced, sequence variation among species seems considerable, so that the rat clones will be useful. The rat brain cDNA library will be a source of cDNA clones for other nervous systems genes.

C. Essentially nothing is known about the control of enkephalin biosynthesis. The methods employed in this study can be used to study this problem. As an example, we had envisioned that one consequence of profound opiate addiction would be a reduction in the enkephalin biosynthesis through a hypothesized feedback inhibition. This was not found; thus, other ideas relating enkephalin to opiate addiction must be entertained.

D. Monoclonal antibodies against various regions of the enkephalin precursor would be useful for biosynthetic and processing studies involving specific immunoprecipitations, and also for immunocytochemistry. The improvements made by us in improving the yield of positive hybridomas will be of use to other workers. Recent disappointing experience of other labs has been that monoclonals against peptides are often of average affinity and of limited use. Our experience with the antibodies, currently somewhat disappointing in view of the large amount of labor involved, will help determine the role of monoclonal antibodies in neuropeptide research.

E. The responsiveness of the AtT-20 cell line to purified CRF means that an excellent system is now available to study the biochemical events involved in stimulus-secretion coupling elicited by a defined physiological hormone (CRF) interacting with a homogeneous population of cells to produce secretion of a defined group of peptides. Molecular aspects of the hypothalmic-pituitary-adrenal axis can be studied under well controlled conditions.

Proposed Course:

A. We plan to study the cell-free translation and processing of human preproenkephalin using mRNA from post-mortem brain.

B. We plan to finish the cloning and sequencing of rat preproenkephalin cDNA in PBR322 plasmids, then reclone it in M13 phage to obtain single-stranded clones suitable for hybridization. In the future, we hope to screen for clones of other neuropeptide precursors.

C. We plan to study regulation of enkephalin biosynthesis using cell-free translation and mRNA blot hybridization techniques. Initially we plan to look at induction of synthesis by chronic treatment with neuroleptic agents such as haloperidol, which are known to elicit a 2-fold increase in enkephalin levels in striatum. Neuroblastoma and NG108-15 hybrid cells synthesize low amounts of enkephalin; these cells may be useful systems to study enkephalin gene regulation.

D. The monoclonal antibodies will be characterized and evaluated for immunocytochemistry and reactivity with the enkephalin precursor.

E. Further work on the mechanism of action of CRF will depend on the availability of manpower to compete effectively with other groups. If possible, we would like to optimize and characterize the stimulation of adenylate cyclase by CRF.

Publications

1. Dandekar, S., and Sabol, S.L. (1982) Cell-free translation and partial characterization of mRNA coding for enkephalin-precursor protein. Proc. Natl. Acad. Sci. U.S.A. 79, 1017-1021.

2. Dandekar, S., and Sabol, S.L. (1982) Cell-free translation and partial characterization of proenkephalin mRNA from bovine striatum. Biochem. Biophys. Res. Comm. 105, 67-74.

3. Sabol, S.L., Dandekar, S., and L.S. Kranzler, (1982) Cell-free translation of enkephalin-precursor messenger RNA from bovine adrenal medulla and corpus striatum, in Regulatory Peptides: Functional and Pharmacological Aspects.(ed. Costa, E., and Trabucchi, M.) Raven Press. pp 175-183.

4. Hook, V.Y.H., Heisler, S., Sabol, S.L., and Axelrod, J. (1982) Corticotropin-releasing factor stimulates adrenocorticotropin and beta-endorphin release from AtT-20 mouse pituitary tumor cells. Biochem. Biophys. Res. Comm. 106, 1364-1371.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00151-12 LBG

PERIOD COVERED
October 1, 1981 - September 30, 1982

TITLE OF PROJECT (80 characters or less)

The Biology of Cyclic Nucleotides in E. coli

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Alan Peterkofsky	Research Chemist Chief, Section on Macromolecules	LBG, NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemical Genetics

SECTION
Section on Macromolecules

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long-term goal of this project is to characterize the processes involved in the regulation of cellular levels of cyclic nucliotides in microbial cells. Our major concentration has involved the enzyme adenylate cyclase, which appears to play the major regulatory role in controlling cyclic AMP levels. The focus of interest at the moment is to construct a library of new strains of E. coli which will allow us to understand better the regulation of adenylate cyclase activity by transport systems.

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Project Description:

Objectives:

The major current objective is to evaluate the mechanism of action of the enzyme adenylate cyclase.

Methods:

Methods involve the construction of strains of E. coli having unusual properties of adenylate cyclase.

Major findings:

In our past studies, it has become apparent that a variety of cellular processes play a role in regulating the activity of adenylate cyclase in E. coli. To a major extent, these processes all involve some degree of communication of the enzyme through the cell membrane. Our most recent studies have referred to this process as a transmembrane signalling system for the regulation of adenylate cyclase activity.

Significance to Biomedical Research:

Cyclic AMP plays an important role as a cellular growth regulator. An understanding of the mechanisms by which this nucleotide is synthesized will make it more feasible to exogenously influence this process, thereby controlling cell growth.

Proposed Course:

Our intention is to enlarge our library of cell cultures with the hope that we will be able to identify strains that overproduce components of the adenylate cyclase regulatory complex. The effect of such overproduction on cellular properties will be investigated.

Publications:

Peterkofsky, A.: Transmembrane Signalling by Sugars Regulates the Activity of Escherichia coli Adenylate Cyclase. In: Schlessinger D. (Ed.): Microbiology-1981, Am. Soc. for Microbiology, Wash., D.C. pp 4-6, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00152-08 LBG

PERIOD COVERED
October 1, 1981 - September 30, 1982

TITLE OF PROJECT (80 characters or less)

Metabolism of Peptide Hormones

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Alan Peterkofsky	Research Chemist Chief, Section on Macromolecules	LBG, NHLBI
OTHER:	Fiorenzo Battaini	Visiting Associate	LBG, NHLBI
	Yoshiyuko Takahara	Guest Worker	LBG, NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Biochemical Genetics

SECTION
Section on Macromolecules

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.9	1.9	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Histidyl-proline diketopiperazine is a metabolite of thyrotropin-releasing hormone. We have found this compound to have some hormone-like activities. A study of the fate of injected radioactive histidyl-proline diketopiperazine after injection into rats, was carried out. The distribution of the compound throughout the body was characterized. The rate of clearance of the peptide from the blood stream into the urine was evaluated. Binding of the diketopiperazine to membrane preparations was demonstrated.

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Objectives:

The purpose of the studies was to localize regions of the rat that have some capacity to interact with histidyl-proline diketopiperazine.

Methods:

Preparations of radioactively labeled peptide were synthesized and injected into animals. Then the fate of the injected material was determined.

Major Findings:

Histidyl-proline diketopiperazine, after injection into rats, was cleared from the circulation biphasically ($t_{1/2}$=1.25 and 33 min.). Unmetabolized histidyl-proline diketopiperazine appeared rapidly in the urine. The longer half-time of clearance of the peptide of 33 min. suggested a tissue reservoir of the peptide. Histidyl-proline diketopiperazine was found to accumulate in adrenal, liver and kidney. Therefore, specific binding of the diketopiperazine to membrane preparations was examined. Such binding was observed in membrane preparations derived from adrenal and liver.

Significance to Biomedical Research:

Histidyl-proline diketopiperazine has been shown to possess a variety of biological activities. An understanding of the mechanism by which these activities are accomplished will help to understand human physiology better.

Proposed Course:

The finding that histidyl-proline diketopiperazine binds specifically to membrane preparations from adrenal tissue suggests that this peptide may play a hormonal role in that gland. This possibility will be explored.

Publications:

1. Koch Y., Battaini, F. and Peterkofsky, A.: [^{3}H]Cyclo(Histidyl-Proline) in Rat Tissues: Distribution, Clearance and Binding. Biochem. Biophys. Res. Commun. 104: 823-829, 1982.

2. Peterkofsky, A., Battaini, F., Koch, Y., Takahara, Y. and Dannies, P.: Histidyl-Proline Diketopiperazine: Its Biological Role as a Regulatory Peptide. Mol. Cell. Biochem. 42: 45-63, 1982.

☆U.S. GOVERNMENT PRINTING OFFICE: 1982-361-132:677