THE NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

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July 1, 1976 through September 30, 1977

NATIONAL INSTITUTES OF HEALTH
US NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
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July 1, 1976 - September 30, 1977
Office of the Director

The mission of the National Heart, Lung, and Blood Institute is to: 1) conduct and support research to increase fundamental knowledge about the cardiovascular, blood, and pulmonary systems; 2) develop and evaluate improved methods of prevention, diagnosis, and treatment for diseases afflicting these systems; 3) encourage application of proven new techniques by the research and medical communities; 4) provide support for the training of research workers, clinical scientists, clinicians, and teachers in the cardiovascular, blood, and pulmonary fields; and 5) inform both the general public and health professionals about research and clinical advances developing out of Institute programs.

Some highlights of progress during the year in the major Institute program areas are briefly described below.

Heart and Blood Vessel Diseases

A major goal of the Institute is to determine whether and to what extent premature illness or death from arteriosclerosis and its manifestations may be reduced by timely interventions against certain risk factors known to increase susceptibility to the disease and its sometimes disastrous consequences, such as acute heart attacks or sudden cardiac death. Three of the most common and dangerous of these risk factors are elevated blood lipids, hypertension, and cigarette smoking--all of them subject to modification. The results of such risk-factor modification are being examined in several major clinical trials.

—The clinical Coronary Primary Prevention Trial is evaluating a cholesterol-lowering diet plus placebo or the same diet supplemented with the cholesterol-lowering drug cholestyramine in reducing morbidity and mortality from coronary heart disease (CHD) among men with Type II hyperlipoproteinemia (characterized by elevated blood cholesterol) but who exhibited no clinical evidence of CHD upon entry into the study. The study, involving 4,000 volunteers, is being carried out at 12 Lipid Research Clinics.

—The Hypertension Detection and Follow-up Program, begun in 1972, involves a study of 11,000 patients with hypertension at 14 participating Centers. Half the patients are enrolled in a stepped-care program, carried out at the Center and involving a highly systematized approach to therapy. The other half were referred for treatment to their regular physicians or other usual sources of medical care. All are being followed for five years from date of entry into the study to ascertain the impact of adequate blood pressure control on morbidity and mortality from cardiovascular disease. Results thus far indicate that patients

in the Stepped Care Group have experienced a greater average drop in both systolic and diastolic pressure than have those in the Referral Care Group, and a higher percentage of the former have achieved diastolic pressures in the normal range.

--The Multiple Risk Factor Intervention Trial, also begun in 1972, studies nearly 13,000 men, aged 35-57, at heightened risk because of various combinations of elevated blood lipids, elevated blood pressure, and/or cigarette smoking. Half are participating in an intervention program directed toward the modification or elimination of these three risk factors; the other half have been referred to their physicians or usual sources of medical care. It is hoped that systematic modification of these risk factors over a six-year period may reduce mortality from CHD by as much as 50 percent.

Other Institute-supported clinical trials are evaluating techniques of secondary prevention or promising new therapeutic procedures in patients who have already experienced clinical manifestations of coronary heart disease.

--The Aspirin-Myocardial Infarction Study, begun in 1974, is assessing the value of regular daily doses of aspirin--an inhibitor of platelet aggregation and, hopefully, clotting complications of heart and blood-vessel disorders--in reducing the threat of recurrent heart attacks and decreasing mortality among patients who have previously experienced one or more heart attacks. The three-year study involves over 4,200 patients and 20 participating Centers. Thus far, patient adherence to the drug regimens has been excellent and follow-up at one year nearly 100 percent.

--The Unstable Angina Collaborative Trial compared the results of medical versus surgical therapy (coronary artery bypass) in 288 patients with unstable angina pectoris. The data indicated that both groups fared equally well, mortality being low and about equal in both groups, though there was a slightly higher incidence of heart attacks in the surgical group. Medical therapy apparently can provide good control in most cases of unstable angina; however, surgery may be the treatment of choice for patients with obstruction of the left main coronary artery or patients with persistent, intractable chest pain.

An important program initiated during 1976 is concerned with identifying forerunners of hypertension that may be identifiable in young people, possibly long before the onset of the clinical disease and when it may well be preventable. Sixteen grants were awarded for genetic and familial studies; "tracking" of blood pressure levels in children; measurement of certain factors during pregnancy and infancy that may be predictors of hypertension during adult life; and studies among children whose mothers had toxemia during pregnancy or hypertension before pregnancy.

Other studies have disclosed that urinary excretion of kallikreins (a group of enzymes seemingly involved in BP control) appears to correlate with blood pressure levels (the higher the kallikrein concentration in urine, the lower the blood pressure and vice versa). The studies have further demonstrated familial aggregation of this trait, suggesting that urinary kallikrein determinations may be useful as a biochemical marker of the presence or absence of a genetic predisposition toward hypertension.

The Institute coordinated the National High Blood Pressure Education Program since 1972. Its goals include alerting health professionals and the general public to the wide prevalence of hypertension; to the fact that it produces few symptoms or none at all and thus may often be present but undetected; to the health hazards of untreated hypertension and to the fact that the disease can easily be diagnosed and readily controlled by a physician, with corresponding health benefits for the patient.

Recent survey data indicate that since the program began operations, total patient visits to physicians for treatment of high blood pressure have increased 49-percent (8% during 1976 alone); new patient visits for HBP treatment have increased 46-percent (13% during 1976); and the percentage of hypertensives unaware of their disease has dropped from 49-percent to 29-percent.

Other developments during the year have included

- evidence that high-density lipoproteins (HDL) confer protection against the development of atherosclerosis: the more robust the subject's HDL fraction, the lower his CHD risk.

- continued accumulation of evidence in non-human primates that atherosclerotic deposits previously induced in these animals by atherogenic diets can be made to regress by lipid-lowering diets and/or drugs.

- continued improvement of angiographic procedures and contrast agents permitting more detailed x-ray visualization of coronary and peripheral arteries. Some of these methods employed image enhancement techniques similar to those employed by the space program in mapping by aerial photography.

- demonstration that hospitalized heart-attack patients who do not develop complications during the early days after onset may safely be discharged as early as the 7th hospital day, with considerable monetary savings to the patient.

Lung Diseases

Lung diseases falling within the scope of Institute programs affect some 14 million Americans, constitute a major cause of illness and disability, cause nearly 46,000 deaths each year, and cost the economy about $18.6 billion a year in lost wages, reduced productivity, and medical expenses.

In seeking more effective means of prevention, diagnosis, and treatment for these disorders, the Institute supports research in the following areas: basic studies on lung development, structure, and function; studies on chronic obstructive lung diseases, such as emphysema and chronic bronchitis; pediatric lung disorders, such as hyaline membrane disease and cystic fibrosis; fibrotic and immunological lung diseases, such as asthma, sarcoidosis, and a number of occupational lung diseases (asbestosis, silicosis, etc.); lung circulatory disorders, such as pulmonary hypertension and pulmonary edema; and respiratory failure.

Environmental factors, such as cigarette smoking and air pollution, are strongly implicated in the development of emphysema. Recent research indicates, however, that cessation of cigarette smoking may not only halt but actually reverse some of the adverse effects of smoking on lung function. In these studies, sharp reduction or cessation of smoking resulted in distinct improvement, as measured by tests determining abnormalities of large and small lung airways. Whether these abnormalities ever clear up completely in ex-smokers remains to be determined, but early results are encouraging.

Cigarette smoking may also be a factor in certain fibrotic lung disorders characterized by proliferation of lung connective tissue and scarring. The main line of defense against the types of injury usually leading to fibrosis is the alveolar macrophage, which engulfs foreign particles entering the lung. However, macrophages from the lungs of smokers were found to contain fibrous inclusions of kaolinite, an aluminum silicate present in cigarette smoke. Kaolinite was also found in interstitial cells of smokers. This raises the possibility that kaolinite, inhaled in cigarette smoke, may be ingested by macrophages, transported by these cells to the interstitial tissues of lung, and deposited there, thus contributing to the development of fibrosis.

White blood cells may also be involved in lung damage resulting from cigarette smoking. A tissue degrading factor, isolated from white blood cells, produced a localized disease resembling emphysema when injected into the dog lung. The factor was found in high concentrations, bound to lung elastic tissue, at sites of lung injury.

A substance called 3-methyl indole, present in cigarette smoke, has been shown in animal studies to exert toxic effects on certain cells of the airways and the respiratory units (alveoli) of lung within 30 minutes of exposure.

Respiratory distress syndrome, also called hyaline membrane disease, is a leading cause of death among the newborn, especially among premature infants. An important factor in development of the disease appears to be lung immaturity at birth. The immature lung is unable to produce sufficient quantities of a surfactant substance needed to keep alveoli and, perhaps, the smaller airways from collapsing during lung pressure changes occurring with breathing. Animal experiments have shown that certain steroids hasten lung maturation and prevent the development of respiratory distress syndrome in lambs or other animal fetuses delivered

prematurely by caesarean section. Studies currently underway are seeking to determine the effectiveness of using one of these steriods (dexamethasone) 24-72 hours before delivery in mothers-to-be, where amniocentesis (sampling and analysis of fluid from the uterus) or the necessity of delivery before term indicates a high probability of respiratory distress syndrome in their newborn infants.

Blood oxygenators, akin to those employed in heart-lung machines used during open-heart surgery, have been tested as a means of combatting acute respiratory failure from various causes in adults. Acute respiratory distress syndrome in adults, treated conventionally, currently carries mortality rates as high as 70-percent. Unfortunately, the blood oxygenators did not appreciably affect mortality rates. For now, at least, the approach appears to have only limited application to this clinical problem.

Blood Diseases and Blood Resources

In this program area, the Institute supports research concerned with

—Thrombosis and hemostasis. These studies range from clotting complications of heart and blood vessel disorders (such as deep venous thrombosis and pulmonary embolism) on the one hand, to congenital or acquired conditions in which the blood fails to clot normally (hemophilia, severe platelet deficiencies) on the other.

—Sickle cell disease. A debilitating, life-shortening illness affecting chiefly blacks, SCD is genetically transmitted. In children receiving the defective gene from both parents, the red blood cells produce an abnormal form of hemoglobin (hemoglobin-S) that, under various conditions, aggregates in the red cell and distorts it into the characteristic sickled shape. Sickled red cells do not flow readily through the smallest blood vessels, often obstructing bloodflow; have impaired oxygen-carrying capacity; and are destroyed much more rapidly than are normal red cells, thus resulting in anemia.

—Hemolytic anemias. These include disorders characterized by accelerated destruction of red blood cells in particular (Cooley's anemia) or more generalized destruction of blood cells of several types (aplastic anemia).

—Blood resources. Studies in this area seek to improve technology relating to the acquisition, storage, fractionation, and distribution of whole blood and blood products. They are also concerned with increasing the safety of blood products for clinical use, either by eliminating hepatitis and other contaminants from blood products or by developing vaccines and the like to confer protection against them.

Deep venous thrombosis and pulmonary embolism, though most often the result of venous inflammation or injury, may also arise as a complication of surgery, especially procedures requiring that the patient subsequently be immobilized for prolonged periods. Recent studies have shown that venous thrombosis and pulmonary embolism may be prevented in surgical patients by administering low doses of the anticoagulant heparin, beginning before operation and continuing the therapy during the recovery period. When carefully controlled, the regimen does not increase the patient's risk of bleeding complications.

The treatment of hemophilia improved dramatically during the sixties with the development of concentrates of antihemophilic factor (AHF, the clotting factor deficient in hemophiliacs) to replace the fresh whole blood or plasma previously used to prevent or control bleeding episodes. Newer AHF concentrates are stable and can be stored at home for prolonged periods without loss of potency. The hemophiliac or members of his family can be trained to administer these concentrates, so that treatment can begin very shortly after injury or development of a spontaneous bleeding episode. The prompt attention received by the hemophiliac in home-care programs usually reduces the amount of AHF needed to bring bleeding under control and also appears to reduce substantially the incidence and severity of complications following the bleeding episode.

In most transfusion situations, the specific blood fraction needed is red blood cells to restore the normal oxygen-carrying capacity of the recipient's circulation. Research continues toward the development of non-toxic chemical substitutes that can perform part of this oxygen-transport function while the patient's blood-forming tissues replenish his depleted red-cell supply. No suitable chemical substitute has emerged thus far, though cell-free hemoglobin has performed well in animal studies. But the advantages of an acceptable substitute would be twofold: 1) it would "stretch" existing blood resources or could be employed in acute shortage situations and 2) it could be sterilized, thereby eliminating the threat of hepatitis or other infections sometimes transmitted via blood or component therapy.

Intramural Research

The Institute's Intramural Research Program blends fundamental research on the structure and functions of the cardiovascular, pulmonary, and blood systems with clinical studies concerned directly with clinical problems posed by the diseases that affect them. Some recent findings from this program are summarized below.

--The high-density lipoproteins (HDL) of plasma are thought to play an important role in transporting various fatty substances out of tissues, and a robust HDL fraction appears to confer some degree of protection against atherosclerosis. However, Institute scientists have observed a new variant of HDL which they have designated HDL_C. The variant appeared in the plasma of animals fed high-cholesterol diets whether or not their

blood cholesterol levels were elevated. Though heavier than low density lipoproteins (LDL) the HDL_C variant behaves very much like LDL in some ways, including binding to the same receptor sites of arterial smooth muscle cells in culture. Since low and very low density lipoproteins are thought to be atherogenic, and the high-density variant apparently behaves so much like LDL, it may be that not all members of the HDL fraction will prove to be "good guys" after all.

—Radionuclide cineangiography, developed by NHLBI in collaboration with other NIH components, is proving to be a versatile and valuable technique for the diagnosis of coronary heart disease and for assessment of abnormalities in ventricular performance resulting from myocardial blood deprivation (ischemia). The procedure begins with the injection of human serum albumin tagged with technetium-99_m into a perpheral vein. Shortly thereafter, a scintillation camera placed over the patient's chest begins to record the radiation emitted as the blood-borne tracer is pumped through the heart. The emissions recorded by the scintillation camera are converted into visual images, collected, and sorted out by a computer, then displayed on a video tube in an endless-loop format representing the continuously beating heart.

The technique permits overall assessment of ventricular performance as well as localization of specific regions of the ventricle that are contracting poorly as a result of ischemia or other factors. The image data and the EKG data recorded and analyzed simultaneously by the computer also permit accurate determination of filling rate, ejection fraction, and other valuable indices of ventricular performance, both at rest and during intensive exercise (on a bicycle ergometer). The technique is safe and causes practically no discomfort to the patient.

Results thus far with the technique have shown it to be far more sensitive and accurate than the exercise EKG in detecting coronary heart disease, if present, in subjects without clinical symptoms.

It has been used to demonstrate that nitroglycerin reduces or normalizes the ventricular dysfunction that nearly always develops with intense exercise in patients with coronary heart disease, whether or not the patient also experiences chest pain. Such dysfunction, due to transient ischemia triggered by exercise or other factors, may produce no symptoms in many patients with coronary heart disease, yet make them highly vulnerable to potentially serious disturbances in heart rhythm. Arrhythmias are the major death-dealing mechanism in sudden cardiac death. The findings thus raise the possibility that nitroglycerin may be of prophylactic value in patients with moderate to severe coronary heart disease unaccompanied by angina pectoris.

—Many surgeons have considered patients over 60 with rheumatic heart disease poor candidates for surgery to replace heavily

damaged heart valves. From their own experience, Institute surgeons report that, at least with respect to aortic valve replacement, the geriatric set has fared as well as younger patients. From 1966 through 1975, they replaced aortic valves in 73 patients over 60 years old. The percentage of hospital deaths and late deaths from heart disease among these patients was no higher than that observed among patients under 60. The extent of improvement in heart performance and relief of symptoms was also similar in both younger and older patients.

—The Reis-Hancock valve, a pig heart valve preserved in glutaraldehyde and mounted on a prosthetic frame for ease of insertion, is favored by many surgeons for mitral or tricuspid valve replacement, both because of its excellent performance characteristics and its very low incidence of postoperative clotting complications, even in patients receiving no anticoagulants. In addition to performance and blood compatibility, the third major consideration in selecting a valve replacement is durability. Unfortunately there are few long-term data to indicate how long a Reis-Hancock valve might reasonably be expected to perform adequately after clinical insertion. NHLBI scientists have studied 51 Reis-Hancock valves removed from subjects dying from other causes than valve failure at varying periods up to 74 months after insertion. In most instances, the gross appearance of the valve was normal, but close morphological and histological examination revealed that the valve gradually undergoes degenerative ultrastructural changes after insertion. These changes, if progressive over prolonged periods, suggest eventual failure of the valve, though present data are insufficient to predict the probable useful life of the prosthesis.

—Fibrotic (or "interstitial") lung diseases account for about 20-percent of all chronic lung disorders. They are often disabling, are largely untreatable, and some frequently kill their victims within five years after the appearance of overt symptoms. These diseases may have many causes—inhalation of toxic substances, excessive radiation exposure, hypersensitivity, etc.—or may have no discernible cause (idiopathic pulmonary fibrosis), but all are characterized by the formation of scar tissue, often throughout the lung.

NHLBI studies indicate that the lung scarring that occurs in idiopathic pulmonary fibrosis is not due to excessive production of collagen by lung fibroblasts or other collagen-synthesizing cells of lung, as had been previously thought. Cells cultured from patients with this disease produced collagen at rates comparable to cells from "normal" lungs. This finding suggests that the problem is not excessive collagen production per se, but rather normal rates of synthesis in the wrong places.

Other studies indicate that, in patients with idiopathic pulmonary fibrosis, the lymphocytes which normally help protect the lung against "foreign" invaders such as bacteria, react to the connective tissue of the patient's lung as though it were foreign and so tend to attack it. Hence immune and cell-mediated mechanisms may play prominent roles in the progressive disruption of lung connective tissue that occurs with this disease.

—When citrate labeled with Gallium-67 is injected into patients with idiopathic pulmonary fibrosis, about 70-percent accumulate the isotope in their lungs. The areas of most rapid and most intense uptake correlate well with the extent and severity of inflammation of affected lung tissues. Hence the Gallium-67 scan can be of value in assessing the extent and following the clinical course of the disease in many patients or in evaluating the results of therapeutic measures.

—Cooley's anemia is an hereditary disorder of hemoglobin leading to premature destruction of the victim's red blood cells. This, in turn, results in severe anemia that at present can be corrected (temporarily) only by repeated blood or red-cell transfusions. The genetic flaw results in one of the two protein chains used in assembling the hemoglobin molecule being made at a much slower rate than the other. The chain in short supply is the beta chain; alpha chains continue to be produced at a normal rate. In the absence of sufficient beta chains to be combined with, the excess alpha chains accumulate, then precipitate in the red blood cell, thereby drastically reducing its lifespan.

The repeated blood transfusions required by victims of Cooley's anemia, coupled with accelerated destruction of their own red cells, leads to the accumulation of iron compounds that the body cannot readily excrete. Deposition of this iron in various organs and tissues may seriously interfere with their functions and, in fact, is responsible for many of the disabling or lethal complications of Cooley's anemia.

Recent NHLBI studies indicate that regular injections of desferrioxamine, an iron-chelating agent that converts iron compounds into forms that are more readily excreted, can prevent further iron overload in Cooley's anemia patients requiring regular transfusion, though most of the iron already deposited in the tissues tends to stay put.

Conclusion

Are federal investments in research directed against heart, blood vessel, lung, and blood diseases paying off in lives saved or usefully prolonged? With respect to the cardiovascular diseases, declining mortality rates in all major CVD categories indicate they are. For example, between 1972 and 1976 the mortality rate for all cardiovascular diseases combined fell by 12.7%. This figure includes decreases of 27.9% for hypertension and hypertensive heart disease, 18.8% for

stroke, 14% for rheumatic fever and rheumatic heart disease, 8.8% for congenital heart disease, and 12.1% for coronary heart disease (CHD).

The decreases in mortality rates for stroke and CHD are especially significant because together they account for the great majority of all cardiovascular deaths. The downturn in the CHD mortality rate is also noteworthy because it represents a relatively recent trend that did not become apparent until the late sixties. (Mortality rates in most other CVD categories have been declining more or less steadily since 1950, or even earlier in a few instances.)

One obvious reason for the decreasing CHD mortality rate is dramatic improvement during recent years in the hospital treatment received by heart-attack patients. The intensive coronary care units now found in nearly all moderate to large-sized hospitals have sharply reduced deaths from arrhythmias, formerly the leading cause of mortality during the early days after acute attacks. Another probable reason is improved medical managment of conditions such as hypertension and elevated blood lipids that increase the individual's risk of premature CHD and its manifestations.

More difficult to evaluate, but probably a very significant factor is that large numbers of Americans have voluntarily altered habits and modes of life thought to increase susceptibility to premature CHD. This includes adopting more prudent dietary patterns to keep weight and blood lipid levels in line; eliminating smoking or reducing cigarette consumption; and embarking on sensible programs of regular exercise.

Recent mortality data suggest that we are at last turning the corner in the battle against CHD, far and away the leading killer among cardiovascular diseases.

ANNUAL REPORT OF THE DIVISION OF HEART AND VASCULAR DISEASES, NHLBI

JULY 1, 1976 - SEPTEMBER 30, 1977

1. General Mission of the Division of Heart and Vascular Diseases

The Division of Heart and Vascular Diseases (DHVD) is responsible for planning, directing and conducting the National Heart and Lung Institute's research grant, contract and training programs in heart and vascular diseases. These programs encompass basic research; targeted research; clinical trials; education, demonstration and control activities; and Manpower Training Programs. The Division maintains surveillance over developments in its program areas and assesses the national need for research in the causes, prevention, diagnosis and treatment in these disease areas. The scope of the Division's mission is indicated by the ten research areas which have been identified in the Institute's National Plan and Program: Arteriosclerosis, Hypertension, Cerebrovascular Disease, Coronary Heart Disease, Peripheral Vascular Diseases, Arrhythmias, Heart Failure and Shock, Congenital and Rheumatic Heart Diseases, Cardiomyopathies and Infections of the Heart, and Circulatory Assistance.

There continues to be a slight, but encouraging decrease in the death rate in coronary heart disease, but cardiovascular disease remains the number one killer in the United States. A major focus of the Division is on arteriosclerosis and hypertension which are underlying factors in over 1,000,000 deaths in this country every year.

In studying the ten research areas of the National Plan the Division uses a broad based approach, employing all available funding and administrative mechanisms to take action to support (1) investigator-initiated research, which continues to be the Division's highest priority; (2) clinical evaluation of promising research findings and concepts through clinical trials; (3) targeted research and development focused on problem areas of major importance; (4) education, demonstration and control programs such as the National High Blood Pressure Education Program, and the National Research and Demonstration Center which translate research concepts to practical patient care; and (5) training research manpower in shortage areas.

2. Progress Toward Meeting Objectives of the Division of Heart and Vascular Diseases

Before focussing on progress made in the last year, it may be worthwhile to take a very brief look at the progress made in cardiovascular disease prevention and treatment in the 30 years that have elapsed since the beginning of the National Heart Institute (the forerunner of the Division of Heart and Vascular Diseases in coverage) in 1948.

Over the past 30 years the accomplishments of the Division and the investigations it has supported are legion. In those years, studies of heart disease in the population, diagnostic procedures such as cardiac catheterization, therapeutic interventions such as the cardiac pacemaker and replacement heart valves, devices such as the cardiac by-pass pumping

system, drugs which control blood pressure and lipids as well as cardiac function, have permitted an attack on disease such as has never been seen before. Intracardiac anatomy, function and pathology, as well as vascular disease such as coronary atherosclerosis can be carefully visualized and the heart by-passed at operation so that repairs can now be made under direct visualization. The risk factors of high blood cholesterol, elevated blood pressure and cigarette smoking were identified as being associated with coronary heart disease. The effects of obesity, diabetes, sedentary life style and stressful living patterns were described. Cardiology became identified in every medical school in the country. The ranks of physicians trained in cardiology swelled. Both patients and the public were increasingly able to receive high quality cardiac care in the outpatient and in-hospital areas. During the thirty year period of Division of Heart and Vascular Diseases activity the death rate from cardiovascular disease has dropped by 30%; the sharpest decline has occurred since 1970.

Activities in the Division's targeted areas are proceeding according to schedule. The following summary of the status of the Division's major clinical trials and selected other activities provides evidence of progress toward meeting a number of the designated objectives of the Division.

The Aspirin-Myocardial Infarction Study (AMIS)

In this study, begun in 1974, 30 clinical centers are determining whether the regular use of aspirin by men and women who have had heart attacks will result in a reduction in total mortality. As reported last year, the study completed enrollment of over 4200 patients in the planned one year enrollment period. The first of the three annual patient follow-ups has been completed, with the Policy-Data Monitoring Board periodically reviewing data on endpoints and toxicity. Follow-up and adherence in AMIS have been excellent. As of January, 1977, with approximately 11 months of follow-up, only 2.1% of the participants had missed a scheduled visit, and adherence to prescriptions was at the 95% level. Follow-up is scheduled to continue until the summer of 1979.

The Multiple Risk Factor Intervention Trial (MRFIT)

The primary objective of MRFIT, which started in 1972, is to determine whether, for a group of men at above average risk from coronary heart disease, a special intervention program for six years will result in a significant reduction in mortality from coronary heart disease. The 12,866 men selected for the study, being conducted in 20 centers, were randomly assigned to one of two equally sized groups: (1) a Special Intervention program which is designed to achieve and maintain a lowering of serum cholesterol, a reduction in blood pressure and cessation of cigarette smoking or (2) a usual care group, who have been referred to their physicians or other usual source of medical care. The trial is experiencing different rates of success in achieving reductions in the three risk factors being attempted, with cigarette smoking showing the most reduction, and serum cholesterol the least. Continued attention and efforts are being directed at improving the techniques and the results of intervention.

Hypertension Detection and Follow-Up Program (HDFP)

The HDFP is studying some 11,000 patients in 14 centers who have elevated blood pressures, to determine the effectiveness of a systematic approach of anti-hypertensive therapy in reducing morbidity and mortality. This Program which started in 1972, calls for a follow-up of five years care for each person in the trial. Half of the participants are assigned to a Stepped Care Group which receives a series of carefully controlled drug regimens at the HDFP clinics; the other, the Referred Care Group, are referred to their physicians or other usual source of medical care. Last year the Stepped Care Group showed a drop in the mean systolic blood pressure of 27.9 mm Hg and in diastolic, a drop of 14.5 mm Hg. The Referred Care Group showed a drop in mean systolic blood pressure of 16 mm Hg and in the diastolic, 8.8 mm Hg. About 66% of the Stepped Care Group had diastolic blood presure less than 90 mm Hg, compared with 43% for the Referred Care Group. Approximately 54% of Referred Care Group reported that they were on antihypertensive drugs at this visit, an increase of 2% over the first year.

Unstable Angina Collaboration Trial

This study compared the results of medical vs. surgical treatment of 288 patients with unstable angina. Enrollment in this trial was completed at the end of last year. The report of the data on the 288 randomized patients was presented at the American College of Cardioolgy meeting in February. Analysis of the data revealed that mortality was low and equal in both medical and surgical groups; myocardial infarction occurred more often in the surgically treated group than in the medically treated group. The investigators agreed on a statement calling for admission of such patients into the Coronary Care Unit, intensive pharmacologic therapy with study of the angiographic and hemodynamic data, continued pharmacologic therapy for most patients, and surgery for those with left main coronary artery disease or with continued intractable pain; their statement concluded with the finding that prophylactic surgery to prevent MI and death is not otherwise indicated.

Lipid Research Clinics Program (LRC)

Six Lipid Research Clinics were established in 1971 and an additional six in 1972, eleven of them in the United States and one in Canada. In 1975 an LRC was established in Jerusalem, Israel. Two major sets of collaborative studies were undertaken by the LRC program: population studies and a Clinical Coronary Primary Prevention Trial. The population studies are determining the prevalence of hyperlipidemia and describing the distribution of lipids and lipoproteins in major population groups. The Clinical Coronary Primary Prevention Trial is testing the hypothesis that if blood lipids are reduced, coronary heart disease morbidity and mortality will also be reduced. At a press briefing in July, 1977 a number of findings made by the LRC's to date were announced. Among the key findings were:

- Lipid Distribution - Population based distributions have been established for cholesterol and triglyceride levels. Included is information derived from study of about 20,000 subjects age 0 to 19 years. Blood lipid levels in children are attracting increasing attention as it is recognized that the factors that determine atherosclerosis are probably initiated in childhood. Of special interest in the pediatric age group is a fall in plasma cholesterol in adolescence.

- Effect of Oral Sex Hormones - Women taking oral contraceptives have been found to have substantially higher triglyceride levels and more modest elevation of cholesterol levels than women not on such medication.

- Nutrition - Analysis of one day diet recalls from several thousand participants suggests that there has been a reduction in the U.S. daily cholesterol intake and an increase in the ratio of polyunsaturated to saturated fats. These findings are consistent with other measures of U.S. food consumption and, if confirmed, could explain the blood cholesterol reductions that have been reported.

Coronary Artery Surgery Study (CASS)

This study, started in 1973, has two components: an assessment of the effects of coronary artery bypass graft surgery on morbidity and mortality, based on a registry of patients who have had such surgery, and (2) a prospective, randomized study comparing surgery and medical management. Fifteen sites are participating in the study (14 in the U.S. and one in Montreal). Approximately 17,000 patients have been entered into the registry, which has a goal of 20,000, and 450 patients have been randomized into the clinical trial, for which a total of 600 patients has been projected. The study is scheduled to terminate in 1983.

High Blood Pressure Education Program

There are a number of indications of the impact being made by this program:

- There was an increase of 8% in total patient visits to physicians for treatment of high blood pressure during 1976, compared to a 1% decrease for all visits, resulting in a 49% increase in total hypertension-related visits since 1971 (the base year).

- There was a 13% increase in new patient visits to physicians for treatment of high blood pressure in 1976, and a 46% increase since 1971.

- The percentage of hypertensive persons unaware of their disease dropped from 49% to 29% since 1972.

The Program conducted the Secretary's Conference on High Blood Pressure Control in the Worksetting to explore the possibility of extending education and control efforts for hypertensives in the worksetting. As a follow-up of findings and recommendations made at the Conference, the Program is initiating several contract-supported endeavors along these lines.

In response to Congressional suggestions, the Program is starting, in FY 77, the support in several states of state-wide coordination of high blood pressure control activities. The outcomes will be evaluated through sample surveys of hypertension control status and through changes in selected mortality and morbidity data.

Diabetes and Cardiovascular Disease

This grant program, initiated last year, is designed to encourage increased attention to the relationships of diabetes and cardiovascular disease. Twenty-nine grants have been awarded. Among the recent findings in this area is that the glycosylated minor hemoglobin components Hb^{A}_{a+b+c} are elevated in insulin-dependent juvenile diabetic patients. It may be that measurement of these components can provide improved techniques for screening potential diabetics and for determination of the adequacy of control of blood glucose in diabetics already diagnosed. A second RFA has been issued. Sixty-three applications have been given initial review and will be reviewed at the September meeting of the Council.

The High Blood Pressure in the Young Program

This program, started mid FY 1976, has 16 grants, conducting research in the following areas: genetic and familial studies; studies of tracking of BP levels in children; prospective follow-up studies of factors measured prenatally and during infancy; studies of children of toxemic pregnancies; and other factors, such as diet and psychological growth.

The investigators have had two meetings to discuss matters of common interest, such as protocols, definitions and approaches.

Natural History of Congenital Heart Defects

The Report from the NHLBI-supported Joint Study on the Natural History of Congenital Heart Defects was published in Circulation, August, 1977, Volume 56, Supplement Number 1. It is American Heart Association Monograph Number 53. The report covers the indirect assessment and clinical course of Pulmonary Stenosis, Ventricular Septal Defect and Aortic Stenosis.

3. Highlights of Accomplishments Ocurring Within the Reporting Period

Significant progress continues to be made in meeting the Division's objective of increasing knowledge of the basic mechanisms and processes leading to cardiovascular disease and of closing the gap between this knowledge and the health care delivered to the patient with cardiovascular disease or the person with a high risk of developing such disease.

A few examples:

In atherosclerosis research, the past year has been unusually fruitful in terms of development and verification of new knowledge. Progress is summarized for a number of areas:

- There has been increasing evidence that High Density Lipoprotein (HDL) is an anti-risk factor with epidemiological, clinical and animal experiments indicating that its levels are inversely related to risk.

- A fundamental new concept proposed in recent times is the view put forward about three years ago that atherosclerotic plaques are generally monoclonal, and therefore may represent transformed cells and be generically of the class of (benign) neoplastic phenomena. During the past year two other laboratories have reported data confirming that mature plaques are generally monoclonal or monotypic. They have also determined that fatty streaks are not monoclonal. While thick plaques are monotypic, thin plaques are not. While other explanations can be offered for these findings that matter deserves further study.

- The past year has seen the reaffirmation in several major studies in monkeys that regression of plaques will occur, and the time course of the observed variables has been described.

In Hypertension research:

- A biochemical alteration has now been correlated with elevated blood pressure. Urinary kallikrein excretion, which is significantly reduced in adult essential hypertensives, has been studied in a cohort of children in whom familial aggregation of blood pressure has been demonstrated. Familial aggregation of urinary kallikrein was shown for both black and white children (ages 5-18) analyzed separately. Urinary kallikrein concentration was significantly lower in black children than in white. Families with lowest mean kallikrein concentrations tended to have higher blood pressures than those with highest kallikrein. While this study does not clearly involve urinary kallikrein in the pathogenesis of essential hypertension, it nevertheless suggests a potential relationship between a biochemical marker and blood pressure in children.

- Basic and clinical investigations on the role of mineralocorticoids in hypertension continue to receive much emphasis. Studies are attempting to understand the meaning of elevated plasma concentrations of 18-OH DOC seen in some patients with essential hypertension. Since this hormone has been associated with experimental hypertension, its role in human hypertension may be more significant than originally thought. The observation of an exaggerated natriuresis in experimental and human hypertension unrelated to mineralorcorticoide activity is also receiving attention.

In research involving cardiac function:

- Attempts to evaluate the interrelationships between the autonomic nervous system and the conduction system of the heart in the production of dysrhythmia have led to the development of the dog model in which the SA node has been chronically excised. In the conscious dog with the intact SA node, there is normally a marked sinus arrhythmia closely coupled to respiration. The animal with the excised SA node has no phase relationship between cardiac and respiratory rhythms but rather develops a periodic ectopic rhythm disturbance that is reproducible. This arrhythmia is associated with changes in autonomic tone that may be linked to baroreceptor stimulation (since the dysrhythmia is associated with marked changes in blood pressure). Efforts are continuing to study these interrelationships in the production of cardiac dysrhythmia.

In cardiac disease research:

- The demonstration of the feasibility of discharging the uncomplicated patient with acute myocardial infarction on the 7th hospital day had led to a program of early discharge of these patients in one IHD-SCOR institution. Preliminary analyses indicate that the average hospital stay has been halved in the overall group of uncomplicated patients (17 days to 8.5 days). In addition to this overall decrease in hospital stay, there has been a saving of approximately $1,000 per patient in patients actually discharged on or after day 10. There has been no significant morbidity or mortality in the post-discharge period.

In the area of devices and technology:

- Continued technology improvements in the therapeutic use of cardiac pacing have been accomplished. These improvements include: refinement of patient treatment and follow-up using electronic and transtelephone communication; development of clinical and research data recall; development of a rechargeable battery-powered cardiac pacemaker; extending the life of cardiac pacemakers by developing new and improved electrodes; and the development of a heart-powered miniature implantable pacemaker. Clinical testing of a rechargeable battery-powered cardiac pacemaker has continued with excellent success. Ten patients currently have such a device.

- New intravenous contrast agents and electronic signal processing techniques are being developed and evaluated to permit improved radiographic visualization of the peripheral and coronary arteries. Enhancement of the contrast of low contrast images has been demonstrated by a subtraction technique derived from aerial photographic mapping. Early results on imaging of the coronaries in dogs are promising, and optimization of the technique is continuing.

4. Significant Workshops, Task Forces and Meeting

A two week Training Workshop on Cardiovascular Epidemiology was held in the Summer of 1977 to provide an intensive, comprehensive introduction to the principles and methods of the epidemiological study of cardiovascular diseases. Twenty selected cardiologists attended the Workshop. A similar one was held in the Summer of 1976.

A Symposium on Vascular Grafts, held for two days in November, 1976, was attended by 350 physicians who heard 39 presentations. The presentations dealt with the current efficacy and shortcomings of a wide variety of prosthetic graft materials used in peripheral arterial and coronary arterial reconstructive surgery. Publication of the presentations is in process.

Forty participants, predominantly from the targeted programs of the Division, met in a workshop in November, 1976 to assess critically the developments in isotopic imaging related particularly to characterizing infarct size and zone of ischemic involvment. The meeting was held immediately prior to the Annual Scientific Meeting of the American Heart Association.

Another meeting held just before the Annual Scientific Meeting of the AHA, had 25 participants, mostly from targeted programs of the Division, reviewing the current methodology and status of enzyme techniques for the quantification of the extent of myocardial infarction.

The Division's Task Force on Hypertension, established last year to survey the entire field of hypertension research and to identify areas needing intensified or lessened emphasis, held several meetings this year. Its report is due in the Fall of 1977.

A Task Force on Heart Disease in Childhood, established earlier this year, has held several meetings and is proceeding on schedule in surveying needs and opportunities for research and prevention in this important area. Its report will be ready in January, 1978.

A Task Force on Prevention and Education has been established with the goal of identifying areas which are amenable to prevention and/or education and recommending to the Institute methods and research programs to implement prevention and education in those areas. The Task Force is beginning its deliberations in September, 1977.

A second printing was made of "Proceedings of the Nutrition-Behavioral Research Conference" held in 1975. It is available as DHEW Publication No. (NIH) 76-978. To date more than 1700 requests for this publication have been filled.

The Report of the Task Force on Blood Pressure Control in Children was published as a supplement in the May, 1977 issue of Pediatrics, Volume 59, Number 5. Copies of the Supplement are available from the National High Blood Pressure Education Program, NHLBI, Bethesda. The Report covers Recommendations, Methodology and Instrumentation for Blood Pressure Measurement in Infants and Children, Standards for Children's Blood Pressure, Evaluation of Children and Infants with Elevated Blood Pressure, Treatment, Mechanisms and Causes of Hypertension, and includes tables on Mean Systolic Blood Pressure in Children Aged 2 to 18, and a table on Mean Diastolic Blood Pressure.

5. Major Problem Areas

The trend toward "safe but dull" research approved by the review process is a major problem. The trend is manifest by the lack of approved short-term "high risk" research.

Ability to support fifty percent of investigator-initiated grants and at the same time manage a balanced targeted program in the face of current commitments continues to be a major concern.

The slow erosion of support for heart and vascular disease is a primary concern.

The continued difficulty in recruitment of excellent young scientists in the face of budget and personnel limitations is a problem of major importance.

Problems exist in the management of the clinical trials programs:

a) The lack of academic rewards for investigators managing the programs.

b) Premature information acquired by the scientific community jeopardizes the reliability of data being collected and attainment of goals.

c) The inevitable requests for extension of trials in order to assure ascertainment of positive results, as well as justification for such extensions, in the face of budget limitations.

DIVISION OF LUNG DISEASES

ANNUAL REPORT

July 1, 1976 through September 30, 1977

I. MISSION OF DIVISION OF LUNG DISEASES PROGRAMS

The broad program goals of the Division of Lung Diseases are more effective diagnosis, treatment and, ultimately, prevention of pulmonary disease. To these ends the Division emphasizes seven program areas: Structure and Function of the Lung (includes lung development); Chronic Obstructive Lung Diseases (emphysema, chronic bronchitis, asthma); Pediatric Pulmonary Diseases (neonatal respiratory distress syndrome, cystic fibrosis, bronchiolitis); Fibrotic and Immunologic Interstitial Lung Diseases; Pulmonary Vascular Diseases (pulmonary hypertension, cor pulmonale, pulmonary edema); Respiratory Failure; and Prevention, Control, and Education.

In addition to supporting traditional, investigator-initiated research, program project and training grants, research career awards, and young investigator research grants, the Division has stimulated research or training in neglected areas through special, goal-oriented programs: Specialized Centers of Research (SCOR) Grants support interdisciplinary research with a clinical emphasis. Research and Demonstration Center Grants include demonstration and education projects to prevent and control lung diseases. Pulmonary Academic Awards and Pulmonary Faculty Training Grants and Awards meet special needs relative to pulmonary research and clinical manpower. The Division uses the research contract to foster targeted pulmonary research and development projects.

To implement its programs more effectively, in September 1976 the Division reorganized into four Branches: Structure and Function; Airways Diseases; Interstitial Lung Diseases; and Prevention, Education, and Manpower. This administrative structure now reflects the disease and program categories in the National Plan formulated in 1972.

II. PROGRESS TOWARD OBJECTIVES

For many years a major obstacle to advances in pulmonary research was the failure of basic scientists to perceive the relevance of their disciplines in lung diseases, or to draw upon special features of the lung that could further their basic investigations. To correct this deficiency, the Division has used a variety of approaches to help basic scientists understand lung diseases and to encourage work on pulmonary problems. By issuing requests for applications (RFAs) and for contract proposals (RFPs), the Division has reached investigators not previously concerned with diseases of the lung. The success of this endeavor is evident in the scientific accomplishments highlighted in Section III of this report, and in the fact that the number of regular research grant applications submitted in FY 77 was 52 percent greater than in FY 76. Moreover, many fundamental investigations have progressed to a stage where they are now directly applicable to

diagnosis or treatment, or have elucidated the pathogenetic mechanisms of specific pulmonary diseases. Noteworthy examples are these:

- Investigations of respiratory mucus are now applicable to studies of chronic obstructive lung diseases and cystic fibrosis.

- Advances in sensor technology are now used to assess pulmonary function in infants and children, to measure pulmonary hypertension, and to monitor patients with acute respiratory failure.

- Studies of connective tissue components have contributed to an understanding of emphysema and fibrotic lung diseases.

- Investigations of proteases and antiproteases have elucidated their role in etiology and pathogenesis of emphysema.

Investigator interest in the various types of lung cells has advanced remarkably since 1973 when the Division issued an RFP to stimulate work with the--then new--techniques for lung cell separation and isolation. To meet the needs of the increasing number of researchers who now wish to use these techniques, the Division is sponsoring a course on lung cell culture that will be offered for the first time in September 1977. With regard to the four main structural components of the lung--collagen, elastic fibers, proteoglycans and basement membrane, greatest progress has been made in structural studies of collagen and elastic fibers. Investigations of the other structural components has been encouraged by the Division through issuance of an RFA on lung proteoglycans, and sponsorship of a workshop on lung basement membranes.

Since 1970 the Division has supported epidemiologic studies to identify risk factors for chronic obstructive lung diseases. A large number of populations with identified characteristics are now available for longitudinal studies. To capitalize on this resource, a collaborative, prospective study has recently been initiated to determine whether individuals known to be heterosygous (MZ phenotype) for alpha-1-antitrypsin deficiency are predisposed to chronic obstructive lung disease, as is known to be the case for homozygous (ZZ phenotype) individuals.

Although the cost and duration of clinical trials impose limits on the number that can be supported, the Division, with the counsel of the Pulmonary Diseases Advisory Committee, has initiated several studies of regimens or procedures that are currently used in medical practice although there is inadequate evidence that they are effective, and some are based on hypotheses of dubious scientific merit. The Division has undertaken an orderly effort to encourage investigations that were recommended at a conference on the scientific basis of respiratory therapy held in 1974.

A controlled clinical study of 12-hour nocturnal oxygen therapy is underway at six institutions to compare this mode of administration with continuous (24-hour) oxygen. In response to an RFA issued by the Division, five in-

stitutions are now investigating problems associated with aerosol therapy. Intermittent positive pressure breathing (IPPB), one of the most costly and widely used modes of respiratory therapy, is the subject of a clinical trial at five institutions and a data center. Because of the complexity of this undertaking, the protocol is still under development and it will be some months before the trial begins. A fourth controlled trial is to assess the benefits in terms of reduced incidence of neonatal respiratory distress syndrome when dexamethasome is administered to the mother 24 to 72 hours before parturition. Five collaborating institutions and a coordinating center are admitting and storing data on the first patients.

A completed trial of extracorporeal membrane oxygenation (ECMO) is referred to in Section III (see Respiratory Failure) of this report. A workshop on the mechanisms of acute respiratory failure, sponsored by the Division, emphasized that the problems of adult respiratory distress syndrome should be approached through interdisciplinary investigations of the mechanisms of acute lung injury. In the opinion of the Division and its Advisory Committee, a new SCOR competition is the appropriate way to implement this workshop recommendation.

Bilateral carotid body resection, a therapeutic procedure that has engendered considerable controversy, is being performed on about 100 patients a year in this country. At issue is whether the patient who has failed to respond to medical modes of treating asthma or emphysema is helped by the surgical procedure, as is claimed by proponents of bilateral carotid body resection. But of even greater concern is whether removal of carotid body innervation, which is involved in control of respiration, may have harmful effects under certain stressful conditions. Because of the claims and counterclaims, the Division has constituted a Task Force that is developing a protocol suitable for studies that could answer some of the controversial questions.

Improvements in management of cystic fibrosis have extended the lifespan of children with this hereditary disorder. As cystic fibrosis patients now often live well into adulthood, many of the problems of therapy are not unlike those of other chronic diseases of the airways. This commonality was the focus of a recent conference on cystic fibrosis and chronic obstructive lung diseases that was cosponsored by the Division and the Cystic Fibrosis Foundation. The clinical studies of aerosol therapy, nocturnal oxygen therapy and IPPB, referred to above, will contribute to the management of cystic fibrosis as well as to chronic obstructive lung diseases. However, a major unresolved problem is to identify genetic markers that will ensure early diagnosis. The Division will soon be awarding contracts for study of ciliary inhibitory factors that are believed to be specific for cystic fibrosis. The underlying mechanisms that are involved in this disease are the subject of a comprehensive analysis of the current state-of-the-art relative to cystic fibrosis, a study jointly supported by the Division of Lung Diseases and NIAMDD.

An important step toward a more effective pulmonary prevention and control program is the issuance of the report of the Task Force on Prevention, Control, and Education in Respiratory Diseases. Now widely disseminated to biomedical and other communities concerned with problems of national health, the report recommends demonstrations and educational activities to bring the

findings of research to bear on medical practice in community settings. While the Division is strongly committed to the recommended program, the rate of implementation will be necessity be dictated by availability of funds. Some recommended activities can be mounted at relatively little cost but others, which involve whole communities, will be more expensive, hence more difficult to undertake.

In another educational approach to prevention and control of lung diseases, the Division issued an RFP to encourage development and evaluation of programs to teach children with asthma how to cooperate in management of their disease. These programs are expected to reduce the need for physician visits or hospitalization. Contracts will be awarded before the end of the current fiscal year.

Division initiatives for manpower development are designed to increase the number of pulmonary physicians who will remain in academic medicine, and to improve the level of knowledge about lung diseases that is provided in medical schools. The eminently successful Pulmonary Academic Award Program has again been announced for competition; awards will begin July 1, 1978. The newer Pulmonary Faculty Training Program, which now support six Training Centers, has been slower to develop. However, it is now making promising strides in attracting trainees from schools that wish to strengthen their pulmonary faculties. A competition has again been announced for medical schools that wish to sponsor a junior faculty member to be trained at one of the Training Centers. Awards will begin July 1, 1978.

III. HIGHLIGHTS OF ACCOMPLISHMENTS

It is difficult in a large program to single out specific accomplishments without doing scant justice to the work of many investigators. Moreover, it is unrealistic to attribute achievements to a given year, as scientific advances are usually the final expression of work pursued over several years and of progress made in small steps. Hence, the accomplishments highlighted in this section are but a sample of the many advances being made in Division-supported pulmonary research, and exclude topics amply covered in prior Annual Reports.

The mechanisms that control ventilation are being examined through studies of the effects of prolonged hypoxia at high altitude, of ventilatory adjustments during exercise, of alterations of respiration during sleep and as a consequence of emotional states, and of responses of patients who have undergone bilateral carotid body resection. A still challenging question after a century of investigation is the mechanism by which ventilation increases so rapidly after onset of exercise, too rapidly to be explained by accumulation of metabolites or transport of blood-borne mediators. Recent investigations have shown that when the exercised limb is occluded, 80 percent of the ventilator response is delayed. While this suggests that the major portion of the response to exercise is through blood-borne mediators, it leaves unanswered the question of how the immediate, rapid increase in ventilation after exercise is mediated. Studies of the very young opposum have shown for the first time that the immature animal shows the same rapid response to exercise

as the mature animal, and that it maintains elevated breathing throughout exercise.

Prolonged hypoxia due to residence at high altitude has been known to blunt chemoreceptivity. Recently, it has been found that neonates who have gestated and been born at altitudes of 3,100 meters lack a chemoreceptor response to hypoxia. However, if gestated at 2,500 meters or below, but born at 3,100 meters, the chemoreceptor response is the same as for neonates born at sea level, and even neonates who lacked chemoreceptor responses at birth acquire normal responses by 15 to 30 days of age.

Of interest in relation to lung development in general and surfactant production in particular, is the finding that animals fed diets deficient in essential fatty acids secrete surfactant in which the amount of palmitic acid in the lecithin moiety is lower than normal. Moreover, this modified surfactant has a less than normal effect in lowering surface tension. These observations, and the fact that infants with respiratory distress syndrome also have much less than normal levels of palmitic acid in the surfactant molecule, suggest that it may be important to determine the quality as well as the qunatity of surfactant in assessing risk of neonatal respiratory distress syndrome.

The question of whether exercise stimulates lung growth has been investigated in Japanese "waltzing" mice, which have a genetic abnormality that makes them extremely active and they spend most of their waking time running in circles. These mice have lungs with volumes and internal structure comparable to their littermates which do not have the "waltzing" defect.

Collateral ventilation, through collateral channels to the lung lobules, may be the most important factor in maintaining gas exchange and preventing atelectasis (collapse) in lung diseases where the small airways are obstructed and direct ventilation (from the bronchioles to the lung lobules) is impaired, as in emphysema, chronic bronchitis, and asthma. Recent studies using the fiberoptic bronchoscope have for the first time shown by direct visualization that collateral ventilation is present in normal human subjects, and appears to be considerably greater in patients with emphysema. In another study, collateral ventilation has been shown to decrease in animals when they do not sigh and to increase after a deep breath. This finding suggests that when patients are encouraged to breath deeply after anesthesia or surgery it may increase collateral ventilation as well as prevent airway closure.

Among numerous investigations of alpha-1-antitrypsin deficiency, two new approaches are of considerable promise. A line of human hepatoma cells that synthesizes alpha-1-antitrypsin is now available, and is being used to study mechanisms responsible for the wide range of serum levels of this antiprotease that occur after alterations in hormone levels, infections and chronic inflammatory processes. These investigations should help answer the question whether alpha-1-antitrypsin deficiency is a defect of synthesis, secretion, or both.

The other new approach, which is of potential value is the development of screening tests for human phenotypic variants, is the use of antibodies to

probe the structure of alpha-1-antitrypsin. In the rhesus monkey antibodies have been raised against a phenotypic variant that, according to immunochemical analysis, is 60 percent similar to human alpha-1-antitrypsin.

With regard to lung injury, there is an increasing body of evidence that the white blood cell as well as the lung alveolar macrophage may be involved in the lung damage that results from cigarette smoke. A tissue degrading factor from white blood cells has recently been isolated, purified and shown to lead to a local disease that resembles emphysema when injected in dog lung. Moreover, microscopic studies of the factor have shown it to fix to elastic lung tissue at the site of injury.

Effective mucociliary clearance, which is compromised in chronic obstructive lung disease, has recently been shown to depend on an optimal rate of flow. At either faster or slower rates, clearance of the respiratory tract is less effective. It is now possible to make direct observations of the velocity of mucus flow by introducing teflon discs into the trachea and insufflating them through the inner channel of a fiberoptic bronchoscope. The technique has been used to study patients with cystic fibrosis or chronic obstructive lung disease.

A naturally occuring disease in cattle that resembles emphysema can be prevented when a specific substance, 3-methyl indole (3MI), is prevented from forming or its effects inhibited. Produced from tryptophan in the rumen of cattle and in the intestine of goats and sheep, 3MI is also present in cigarette smoke. It has been shown in animal studies to have a toxic effect on specific cells of the small airways, and on the alveoli, within 30 minutes of exposure.

Two recent studies are of interest because their possible use in detection of diseases due to pollutants in occupational environments. In one investigation it has been shown that the white blood cells of patients with beryllium lung disease undergo characteristic microscopic and biochemical changes when exposed to beryllium in tissue culture. On the other hand, the white blood cells of exposed beryllium workers who do not have lung disease do not show these changes. This finding provides the basis for a test that could be useful in diagnosis and management of beryllium lung disease. In another investigation, the rate of disappearance of a small amount of radioactive aerosol provides a measure of the integrity of the tissue barrier between blood and air in the lung. The rate of disappearance of the isotope is considerably accelerated when there has been past exposure to occupational dusts. These observations may provide the basis for early detection of lung damage in individuals exposed to occupational environments potentially hazardous to the lung.

Among other observations of possible clinical importance is the finding that drugs such as aspirin and indomethacin, which are used to prevent premature labor, cause high pulmonary pressure in animals by increasing resistance to blood flow through the lung. On the other hand, prostaglandins E have a dilator effect, reducing resistance to pulmonary blood flow, while prostaglandins F have adverse effects, increasing pulmonary arterial pressure. Of potential importance in treatment of pulmonary hypertension is the recent

identification of a new class of prostaglandin compounds--the prostacyclins--that have a marked vasodilator activity.

In treatment of pulmonary edema, positive endexpiratory pressure (PEEP) has been used to improve arterial saturation and was believed to have a "drying" effect on the lung. However, recent animal studies have demonstrated that PEEP actually aggravates the problem of pulmonary edema by increasing the water content. This observation suggests the need for reevaluation of the efficacy of PEEP in clinical management of pulmonary edema.

A study of extracorporeal membrane oxygenation (ECMO), now completed, has shown that ECMO as an adjunct to conventional modes of therapy provides no better survival rates than conventional therapy alone. At present the management of adult respiratory distress syndrome is at best supportive and is associated with high (about 70%) mortality. However, the data from the ECMO trial, when completely analyzed, will provide clues to issues which should be addressed in the future through more fundamental approaches.

IV. REPORTS OF WORKSHOPS, MEETINGS, OTHER

A. Workshop Reports

Prostaglandins and the Lung (distributed in FY 77)
Workshop held January 21-22, 1976 in Airlie, Virginia

Isolation, Characterization, and Mechanism of Action of Proteases and Antiproteases (distributed in FY 77)
Workshop held February 9-11, 1976 in Airlie, Virginia

Bronchiolitis (distributed in FY 77)
Workshop held June 28-29, 1976 in Bethesda, Maryland

Mechanisms of Acute Respiratory Failure (distributed in FY 77)
Workshop held October 20-21, 1976 in Arlington, Virginia

B. Other Reports

Respiratory Diseases: Report of Task Force on Prevention, Control, and Education; DHEW Publication No. (NIH) 77-1248, March 1977

Analysis of Current Pulmonary Research Programs: Fiscal Year 1975 (distributed in FY 77)

Division of Lung Diseases Contract Program: July 1976 (distributed in FY 77)

V. MAJOR PROBLEM AREAS

A. Funds for a Prevention and Control Program

A problem that is not new, but has assumed greater importance since the Task Force on Prevention, Control, and Education in Respiratory Diseases

has presented its recommendations, is the absence of funds specifically appropriated for a program that was authorized in the Act of 1972.

To the greatest extent possible, the Division of Lung Diseases has drawn upon funds for research to develop a prevention and control program. However, any further development would compromise the research grant program, unless additional funds are made available for the prevention and control effort.

B. Grant Assignment

A problem that continues to impede development of a program consonant with the Division's mandate under Public Law 92-423 is the absence of appropriate procedures for assignment of applications that are submitted in response to initiatives of the Division, such as RFAs. Although conceived as part of the Division's National Program, applications in response to such DLD solicitations are frequently assigned to other Institutes. This makes it impossible to develop a forward-looking program that is responsive to national needs and consonant with the Division's mandate.

It is of great concern that this problem is essentially unchanged. As yet, no definitive, agreed-upon rules for RFAs have been developed. Moreover, the revision of the Referral Guidelines, initiated by DRG in April 1976, has not been completed. Discussions with other Institutes have not resolved some of the most fundamental problems.

ANNUAL REPORT

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

July 1, 1976 Through June 30, 1977

The programs of the Division of Blood Diseases and Resources seek to improve the diagnosis, prevention, and treatment of blood diseases and related disorders; to assure the efficient and safe use of an adequate supply of high quality blood and blood products; and to promote the application of tissue and organ transplantation through a better understanding of transplantation biology. They deal with four major areas of blood diseases and blood resources: Thrombosis and Hemostasis, Sickle Cell Disease, Disorders of the Red Blood Cell, and Blood Resources. These programs encompass fundamental and clinical research, targeted applied research, professional development and training; and prevention, education and control activities. The aim at all times is the rapid, but orderly, application of new knowledge to health care. To maximize scarce resources, both financial and human, the Division actively coordinates its efforts with federal and nonfederal programs involving blood. In developing and supporting its programs, the Division of Blood Diseases and Resources seeks to use all available support mechanism as appropriate. These include investigator-initiated research projects, program project grants, goal-oriented centers such as Specialized Centers of Research (SCORs), a National Research and Demonstration Center, and targeted research and development contracts. A brief description and some highlights of accomplishments are mentioned below.

THROMBOSIS AND HEMOSTASIS

Hemostasis is the term used to describe the maintenance of a normal fluid state of blood in the vascular system. The numerous factors involved in hemostasis include those leading to fibrin formation, lytic enzymes which digest formed clots and which may be involved in the catabolism of coagulation factors, cellular components of blood such as platelets, and the blood vessel wall which interacts with or synthesizes coagulation and lytic factors. Imbalances in the hemostatic mechanism can give rise to an array of disease conditions which vary from hemorrhagic disorders to thromboembolic disorders. (1) Thromboembolic Disorders: Major goals are to improve diagnosis and therapy so as to bring about prevention through laboratory and clinical investigations, and to bring about further refinement of fundamental knowledge and continued translation to improve care at the bedside. (2) Hemorrhagic Disorders: To improve diagnostic techniques and specific treatments for hemophilia and other bleeding disorders as well as to understand the genetic and pathological mechanisms underlying those disorders. In acquired disorders of hemostasis, research is directed toward better techniques for detecting patients at risk, particularly candidates for major surgical procedures. (3) Platelet Disorders: To improve understanding of congenital and acquired platelet disorders which will provide more effective therapy while also illuminating the role of platelets in the mechanism of thrombosis

and hemostasis.

A number of new, non-invasive methods for the diagnosis of in vivo thrombus formation or hypercoagulability are being developed or evaluated. Radiolabeled fibrinogen scanning is in widespread use and most methods are evaluated in relation to this method and to contrast venography. Currently, one of the most promising tests involves measurement of plasma inhibitory activity towards thrombin or Factor Xa. Using this test, it has been shown that an increased incidence of thrombosis is associated with a decrease in the Factor Xa inhibitory activity. Several laboratories are engaged in studies of the cellular and molecular defects which give rise to von Willebrand's disease. These efforts include attempts to correct the defect in animal models through transfusion of specific plasma fractions from normal animals, investigations of the mechanisms of action of ristocetin and studies of the von Willebrand's receptor in platelets. Observations suggest that von Willebrand's factor may play a role in the genesis of atherosclerosis. Several approaches to study the adhesion of platelets to collagen, release of platelet intracellular components and the aggregation phenomenon are underway. These include the binding of collagen and ADP to intact platelets and their membranes, characterization of the collagen binding sites, studies of the structural requirements for the collagen molecule, the involvement of membrane enzymes in aggregation, and studies of the influence of the vascular endothelium on aggregation. Further studies involve the relationship of prostaglandin synthesis to platelet lipid composition and the physiological significance of prostaglandins and thromboxanes.

RED BLOOD CELL DISORDERS: (Exclusive of Sickle Cell Anemia)

This program is concerned with the development of new knowledge in the areas of Cooley's Anemia, aplastic and refractory anemias, as well as hemolytic anemias. (1) Cooley's Anemia and Other Hemoglobin Disorders: To improve patient treatment and extend the life span of afflicted individuals as well as provide improved quality of life to victims of this disease. Also to elucidate further basic defects, both genetic and biochemical, and provide eventually a complete cure or method of prevention. To develop an understanding of the basic pathogenic mechanisms underlying all aspects of the hemoglobinopathies, other than Cooley's Anemia, and to develop an understanding of the mechanisms of control of oxygen transport. (2) Erythropoiesis: To apply the knowledge gained from studies on erythropoiesis to the cure of stem cell disorders such as aplastic anemia and other disorders of red blood cell production To obtain adequate supplies of this valuable hormone for clinical use (3) Red Blood Cell Membrane and Enzyme Systems: To determine the structure of the red blood cell membrane and elucidate the metabolic systems of the erythrocyte to provide information which may be utilized to improve the health status of patients afflicted with the various congenital and acquired hemolytic anemias.

An RFA on "Improved Methods for the Clinical Management of Thalassemia" was issued to focus resources, facilities and manpower on clinical problems and to expedite development and application of improved methods of treatment. These studies include the evaluation of the iron chelator desferrioxamine to

combat the iron overload resulting from multiple blood transfusions in these patients.

A contract program has been instituted for collection of erythropoietin and development of methods to achieve highly purified hormone. Previously most of the erythropoietin supply came from South America.

BLOOD RESOURCES AND TRANSPLANTATION

This program consists of six sub-program areas. (1) National Blood Policy: Assure the accessibility of an adequate supply of high quality blood and blood products to everyone in need through studies of blood resource management and the establishment of national blood data systems and make recommendations concerning the structure and function of the national blood resource system. Improve the management of our national blood resource through studies of currently operating blood procurement procedures, donor recruitment strategies, current manpower training needs, dynamics of regional supply systems and methods of promoting the safety of blood service operations. Promote more effective planning in the management of the national blood resource through the collection and analysis of national blood resource data. (2) Hepatitis: Prevent the morbidity and mortality from post-transfusion hepatitis and other transfusion-transmitted infections. (3) Safety of Blood Therapy: Eliminate problems related to blood compatibility, toxicity, and sterility encountered in whole blood and blood component therapy and identification of donors and recipients. (4) Blood Component Therapy: Develop clearer indications for the use of blood components and better methods to assess their effectiveness, thereby reducing the use of whole blood. Improve methods of blood separation and preservation. (5) Transplantation Biology: Provide resources for the study of immunology and genetics of transplantation with elucidation of the major histocompatibility complex in man for improved clinical application. (6) Blood Substitutes: Develop a clinically useful blood substitute to supplement and conserve natural blood and blood products.

In addition to assisting in the creation of the ABC, the Institute is supporting research conducted by ABC Task Forces in the following areas: The Regional Association (charged with the development of standard and machine-readable labels for blood products and of other automated methods), Donor Recruitment and National Data System.

A federal initiative to upgrade hemophilia care in the U.S. is the Hemophilia Programs part of Public Law 94-63. Because there were not adequate data that would permit the analysis necessary for implementation of the section of this law on Blood Separation Centers, the Institute supported a study to obtain information that would assist in determining optimal ways of spending the funds available. The overall conclusion of the study is that the demand for Factor VIII preparations will grow at a moderate rate over the next five years and that the supply in each year will adequately meet the demands.

To fulfill its mandate on the management of the national blood resource, the Institute sponsored a national conference on the management and logistics of blood banking in Troy, Michigan on June 9-10, 1977. The major theme of the conference was the impact of systems and financial management on blood centers.

A collaborative study on transfusion-transmitted hepatitis has shown that hepatitis B has virtually been eliminated as a complication of blood transfusion but that so-called non-A, non-B hepatitis remains a serious problem. It has further shown that the epidemiological pattern of transfusion-transmitted non-A, non-B hepatitis is similar to that of hepatitis B, that is, this form has a significantly higher risk of being carried by commercial donors as a class, than by voluntary donors. At the present time, no specific diagnostic method for non-A, non-B hepatitis exists, but it is likely that the increasing utilization of voluntary donors will serve to lower the incidence of the disease in recipients of blood and blood products.

SICKLE CELL DISEASE

The mission of the National Sickle Cell Disease Program is to devise methods to reduce the morbidity and mortality of sickle cell disease through: research and development, both at the fundamental and clinical level; the initiation and expansion of community education, screening and counseling programs, the education of medical and allied health professionals the strengthening and expansion of the base of black professional and technical personnel; and the improvement of care for patients with sickle cell anemia. (1) Comprehensive Sickle Cell Centers: This program component encomposes research (basic and clinical), clinical trials, clinical application, training and community demonstration projects. These Centers focus resources, facilities and manpower in a coordinated approach to sickle cell disease, bridge the gap between research and service and bring findings in one area into practical use in the other. Specific research projects being carried out in the Centers include those related to molecular, cellular, tissue and organ, studies in sickle cell diseases as well as clinical trials and training and education of community persons in methods of screening and counseling. Community service activities include demonstration projects in community education, screening, counseling and rehabilitations. (2) Education: The focus of the Education Program is directed towards the community on a nationwide basis to provide accurate information about sickle cell disease and correct misinformation about sickle cell trait. A multiplicity of educational approaches and techniques are used. They are: consultation and technical assistance, speeches, program planning and development, coordinating of resources, making referrals, preparation of materials, reports and surveys, responding to inquiries and congressional requests, dissemination of information, conducting exhibits at conventions and conferences and working with community groups. (3) Mission-Oriented Research and Development: This contract-supported component has as one of its objectives the study of mechanisms for the supported and treatment of sickle cell disease. Contracts are supported which address the problem of the confirmation of the basic molecular structure of sickle hemoglobin, the sickling phenomenon, trials of anti-sickling agents, prenatal and other diagnostic techniques, and means of treating the various complications of the sickling process.

An RFP has been initiated to identify the frequency of occurrence and concurrence of health related events in the clinical course of patients

with sickle cell disease by prospective longitudinal and cross sectional studies to identify and evaluate the factors which determine the clinical course and the presence or absence of complications of sickle cell disease.

Basic investigations are directed at the regulation of production of different polypeptide chains. Particular emphasis is on the synthesis of fetal hemoglobin in human erythroid cells. The ultimate goal of determining "switch" mechanism is to increase synthesis of hemoglobin F in individuals with severe sickle cell syndromes, since this is known to ameliorate the disease. Seven (7) grants were funded after an RFA to stimulate research in this area.

PROFESSIONAL TRAINING AND DEVELOPMENT

As a focus for the coordination of blood research training, the Institute cooperates with other NIH Institutes, Societies, and with Governmental Agencies to carry out a continued assessment of the national personnel needs for research in blood diseases and blood resources. The Institute currently supports 162 trainees in the Blood Diseases and Resources area. The decrease from the previous year (180) results from the phasing out of "old" fellowships and training programs.

NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
DIVISION OF EXTRAMURAL AFFAIRS
Annual Report
October 1, 1976 - September 30, 1977

The Division of Extramural Affairs is responsible for formulating and coordinating Institute-wide policies 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. It also represents the Institute on overall NIH extramural grant and collaborative program policy committees, coordinates such policies within NHLBI, and coordinates 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 processing services for the Institute, (b) reports and statistics related to the Institute's extramural and intramural programs, (c) initial scientific and technical merit review of competing grants and research contracts for the Institute, and (d) committee management for the entire Institute.

The Division has continued 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. As demonstrated throughout the year, this Council review is quite efficient. The Council meets at least four times a year. Several Council working groups have been established and are active.

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 grants and research contracts.
2. Management functions for grants and contracts.
3. Central storage and maintenance of official files for all grant programs.
4. Obtaining all final reports and accomplishing the close out of terminated grant files.
5. Preparation of review materials for Council, staff, and review committees.
6. Preparation of official and summary minutes of Council actions.
7. Operation of the Policy and Procedures Office.
8. Committee management functions.

The initial technical merit review of research grant applications and research contract proposals has continued to result in markedly increased responsibilities. The types of reviews in the grant program included:

Clinical Trials
Conference Grants
Arterio SCOR Grants
Pulmonary SCOR Grants
Comprehensive Sickle Cell Center Grants
Thrombosis SCOR Grants
Medical School Pulmonary Training Awards
National Pulmonary Faculty Training Awards
Pulmonary Academic Awards Program Project Grants
Young Investigator Research Grants
Hypertension in the Young Grants
Vascular Complications of Diabetes Grants

In addition, twenty-two contract reviews were held directly related to new RFP's issued by the Institute, and twenty-four renewal contract reviews were held. Thus, a total of 46 review committees were convened to review approximately 375 contract proposals during Fiscal Year 1977. Furthermore, approximately fourteen mail reviews, related to unsolicited or sole source proposals, were conducted by the Review Branch. The use of automated typing equipment proved invaluable in helping the Branch accomplish its responsibilities.

The Grants Operations Branch was responsible for approximately 2,600 awards, with a dollar value in excess of $280,000,000 during Fiscal Year 1977. The Branch's responsibilities included the actual issuance of the awards as well as the day-to-day administration of fiscal and procedural matters relating to those awards. The Branch was also actively involved in site visits, Council preparation, and grant budget negotiations, as well as serving as an important interdivisional coordinator. Senior representatives of the Branch continued to be most effective in assisting NIH-OD staff in the development of NIH grant policy and procedure. The Branch's continued efforts to improve its management practices and procedures have undoubtedly resulted in vast dollar savings for the Institute.

The Processing Section of the Grants Operations Branch not only maintained all files (pending, active, terminated) but initiated and checked all encumbrance lists. The section also received, reviewed, and filed or appropriately forwarded items which pertained to NHLBI grants and awards. This included questions about grant policies as well as many forms (activation, appointment, termination, service time, annual payback agreement certification) which related to the Training and Manpower programs.

The Contract Operations Branch was responsible for the administrative aspects of the NHBLI contracts program, which included presolicitation, solicitation, participation in evaluation of proposals, determination of competitive range, negotiation, award, postaward administration, termination, and close-out of completed contracts awarded by the Institute. During Fiscal Year 1977, the size of the NHLBI contracts program remained relatively stable. An increase in the total programs within the Division of Heart and Vascular Diseases was somewhat offset by a slight decrease in Division of Blood Diseases and Resources' activities. During the

last year, the Federal Government's fiscal year end was changed from June 30 to September 30. Contracting during the transition period went quite smoothly and contract programs continued with no disruption. The contracts program was comprised of approximately 500 contracts, representing approximately $90,000,000 during Fiscal Year 1977. This Institute's decentralized operation in contracting continued to prove its worth. The Branch staff continued to operate in a highly professional manner throughout the fiscal year.

The Reports and Evaluation Branch continued to develop procedures that provided the Institute's extramural, collaborative, planning, and budgeting staff with financial and programmatic information on grants and contracts. A contract information system was developed that is available on-line using a Time Sharing Option (TSO). A direct tie to the central NIH accounting system was set up for getting Indirect Cost data on grants that is more timely and in a manner that reduces the end of the year workload. The Branch, actively involved in Council preparation, as well as financial and programmatic tracking of the Institute's programs, maintained and operated an ADP system that is second only to the DRG IMPAC system at NIH. The Branch system contains approximately 15,000 records with over four million data items. Institute expenditures of approximately $300,000,000 in Fiscal Year 1977 were identifiable in terms of the major programs (Heart, Lung and Blood), subprograms within these and areas of research that cross programmatic lines.

The Division of Extramural Affairs demonstrated an active support of the Institute's "Retraining Program" by agreeing to accept the responsibility of retraining all three Institute employees selected for this program. The goal of the program is to offer "dead-ended" employees with high potential, an opportunity to learn new skills that may open the door to future advancement in a professional series. One individual underwent training in the Grants Operations Branch, and two others received training in the Contracts Operations Branch. These were the first Institute employees to be selected for this program, and the Division's decision to support the program has proved mutually beneficial to all. Other Institute employees now have proof that the program actually works; the Institute will soon gain three well trained individuals, capable of providing valuable assistance in the administrative management of Grants and Contracts, and the once "dead-ended" employees will have a much improved opportunity for career advancement.

The Freedom of Information Coordinator responsibility was transferred from the Division's Office of Policy and Procedure to the Executive Office, NHLBI. This relieved the Deputy Director, DEA of a time consuming overload and allowed him to devote more time to other matters relating to policy and procedure. However, the Division still remains a primary resource to both the Freedom of Information Act and Privacy Act Coordinators.

The workload relating to Council preparation has continued to grow so that even the increased space allotted for that purpose was quite cramped. Materials, which might have to be revised, cannot be kept for more than

one post Council round. The still new expanded review cycle has overlapped handling of Council material so that such material for three Council meetings must be received, stored and prepared at one time. This complicated the operation of the Division's Council preparation activities considerably. A large number of summary statements continued to be late, even in the expanded review cycle. In order to get Council material mailed, even two weeks prior to the meeting, we had to duplicate numerous summary statements on our own facilities. This resulted in much use of staff overtime, as well as placing an extremely heavy burden on our equipment. We have yet to find a workable solution to this problem.

Overall, the Division now has adequate space in the Westwood Building to perform the missions carried out there, although this space is spread over four floors which causes considerable inconvenience. However the move from the Landow Building to the Federal Building provided the Contracts Operations Branch with much needed additional space.

In spite of an increasing workload, the staff has continued to perform in excellent fashion and morale appears good. Staff turnover has not exceeded the expected level and we have managed to fill most vacancies. Some additional positions for understaffed areas within the Division would be most welcome and would serve to improve efficiency and morale.

INTRAMURAL RESEARCH

THE NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

ANNUAL REPORT

July 1, 1976 - September 30, 1977

INTRAMURAL RESEARCH

Project Reports

Laboratory of Biochemistry

Laboratory of Cell Biology

ANNUAL REPORT
LABORATORY OF BIOCHEMISTRY
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
July 1, 1976 - September 30, 1977

(A) Cellular Regulation of Enzyme Activity.

(a) Theoretical Analysis of Interconvertible Enzyme Cascades. The singular importance of enzyme cascades in cellular regulation is evident from the increasing number of reports showing that covalent modification of enzymes is involved in the modulation of diverse biological functions, viz intermediary metabolism, protein synthesis, cellular differentiation, membrane functions, chemotaxis, hormone action, and neurochemical phenomena. As noted in last year's report, a theoretical analysis of steady state functions disclosed the extraordinary superiority of interconvertible enzyme cascades over other biochemical mechanisms as a means of regulating key enzymic processes. This theoretical analysis has now been extended to examine kinetic as well as the steady state characteristics of enzyme cascades. The study shows that in addition to serving as amplifier systems with respect to primary signal response and to catalytic potential, cascade systems can serve as multiplier systems with respect to overall rates of catalysis. With respect to time, the overall rate of enzyme catalysis increases exponentially with the number of forward steps in the cascade; therefore, under physiologically feasible conditions a three step cascade can generate high overall rates of catalysis within the millisecond time range.

(b) The Glutamine Synthetase Cascade. (i) In vitro test of the monocyclic cascade model theory. Theoretical studies show that the capacity of adenylyltransferase (Atase) to catalyze adenylylation (inactivation) and deadenylylation (activation) of E. coli glutamine synthetase (GS) is specified by the mole fractions of the unmodified form (P_{IIA}) and the uridylylated form (P_{IID}) of the P_{II} regulatory protein, and also by allosteric interactions that effect rates of the adenylylation and deadenylylation reactions. Studies with purified preparations of GS, Atase and P_{II} protein, show that the steady state fraction of adenylylated GS can be either a parabolic, linear or hyperbolic function of the mole fraction, $P_{IIA}/(P_{IIA}+P_{IID})$ depending on the relative concentrations of the allosteric effectors, glutamine and α-ketoglutarate. Moreover, for a given mole fraction of P_{IIA}, there is large amplification of allosteric effects on the steady state level of adenylylation, and there is a cooperative response of the state of adenylylation to increasing concentrations of either α-ketoglutarate or glutamine. The results confirm in principle, theoretical predictions of the GS cascade and illustrate the usefulness of this as a model to investigate further the regulatory properties of monocyclic and bicyclic cascade systems.

(ii) Mechanism of the adenylylation and deadenylylation reactions. P_{IIA} activates the adenylylation reaction because it increases the affinity of Atase for the positive allosteric effector, glutamine and indirectly because the binding of glutamine to Atase increases its affinity for ATP. In addition, the binding of glutamine is antagonized by α-ketoglutarate. Other data suggest that Atase is a bifunctional enzyme possessing separate

catalytic centers for the adenylylation and deadenylylation reactions (eg. P_{IIA} does not compete for binding with P_{IID}, nor does adenylylated GS compete with the binding of unadenylylated GS.) This conclusion is supported by genetic studies (with Dr. S. Kustu of the University of California, Davis) showing that a strain of Klebsiella aerogenes with an altered Atase structural gene, gln E, has lost its ability to catalyze adenylylation but retains its ability to catalyze the deadenylylation of GS.

(iii) Regulation of the uridylylation and deuridylylation of P_{II}. The uridylylatransferase and the uridylyl removing enzyme activities copurify through many steps of protein fractionation, but there is differential loss of the two activities during storage or by brief heating at 60°C. These results and the fact that both activities are missing in strains of E. coli, K. aerogenes, and Salmonella typhimurium with an altered gln D gene, suggest that both activities are properties of the same bifunctional protein.

(iv) Use of CIBA chrome F3GA as a reporter group to study ligand-enzyme interactions. From spectral shifts (400-700 nm) elicited by the binding of CIBA chrome Dextran FG25 to a hydrophobic pocket in GS, in the presence and absence of competing ligands, the stability constants of several substrates and feedback inhibitors to the enzyme have been determined. Marked variations in the difference spectra of dye bound to taut, (Me^{2+}-enzyme) relaxed, (Me^{2+}-free enzyme) and dissociated forms of the enzyme have been used to study the effects of metal ions, feedback effectors, pH and urea on the kinetics of subunit dissociation. The fact that ADP competes more effectively with binding of the dye to unadenylylated enzyme than to the adenylylated enzyme, was utilized to develop a procedure for separating hybrid species of enzyme containing different numbers of adenylylated and unadenylylated subunits. The method involves affinity chromatography on CIBA chrome-sepharose columns. A detailed analysis of these hybrid molecules should yield important information concerning the role of heterologous subunit interactions on the properties of glutamine synthetase.

(v) Topographical analysis of ligand binding sites. The spatial relationship between the adenylylated tyrosyl group and the two divalent metal binding sites on GS was determined from the ^{13}C-NMR spectrum of 2-^{13}C-AMP adenylylated enzyme and the ^{31}P-NMR spectrum of fully adenylylated enzyme in the presence of Mg^{2+} or Mn^{2+}, and also from the Co^{2+} induced quenching of the fluoresence emission spectrum of Mg^{2+}-enzyme that had been adenylylated with the fluorescence analog of ATP, 1-N^6-etheno-ATP. The 2-carbon atom of the adenine moiety of the adenylyl group is 13 Å from each of two divalent cation binding sites, whereas the phosphorous atom of the adenylyl group is 11 Å from one and 8 Å from the other metal site. In addition the data show that the adenylyl group is highly mobile, and therefore is probably in an exposed position on the enzyme molecule.

(c) Cumulative Feedback Inhibition. Clear evidence that L-alanine, L-glutamate and D-valine can bind simultaneously to each subunit of GS was obtained from stopped-flow experiments in which the changes in intrinsic protein fluorescence associated with the binding of these ligands, both

singly and in pairs, to the enzyme was measured, and also from NMR studies in which the paramagnetic contributions of enzyme bound Mn^{2+} to the proton relaxation rates of bound ligands were measured. These results support earlier conclusions that L-Glutamate, L-Alanine, D-Valine and AMP bind to separate allosteric sites on the enzyme and discredit the report that alanine competes with L-glutamate for binding at a substrate site. The results show further that glycine can bind to both the L-Alanine and the D-Valine allosteric sites.

B. Mechanisms of Enzyme Action.

(a) Theoretical Considerations. (i) From theoretical considerations a new method was developed for distinguishing between various mechanisms of enzyme action. In this method one measures the amounts of a common product formed in reaction mixtures containing various concentrations of two alternative substrates at several different constant ratios. From appropriate graphical treatment of the data the mechanism of the reaction can be ascertained.

(ii) An improved general method for deriving steady state and isotope exchange equations has been developed. The method utilizes two general rules that eliminate redundant terms obtained with older procedures and also eliminates closed-loop terms in complex mechanisms. The improved procedure greatly simplifies mathematical manipulations in deriving rate equations.

(b) E. coli alkaline phosphatase. It has been suggested that the action of alkaline phosphatase involves a flip-flop mechanism in which activities at the catalytic sites on each of two identical subunits are coupled and alternating. In this mechanism substrate binding and phosphorylation at the catalytic site of one subunit must be followed by substrate binding at the catalytic site of a second subunit before dephosphorylation at the first site can occur. This mechanism is contraindicated by a kinetic analysis using the alternative substrate method described above, and also by results of stopped flow experiments showing that the rate of dephosphorylation of ^{32}P-phosphorylated enzyme is independent of substrate concentration.

C. Metabolism of Branched Chain Amino Acids. A functional role in humans of the enzyme, leucine-2, 3-aminomutase, which catalyzes the vitamin B_{12} coenzyme dependent conversion of α-leucine to β-leucine is suggested by the finding that sera from normal humans contain significant amounts of β-leucine. Preliminary studies (on relatively few subjects) indicate that patients with pernicious anemia contain greater than normal levels found in similar patients that had been treated (presumably with B_{12}). The demonstration that cobalamin-dependent leucine mutase is present in tissues of several different plants and in addition that potato tubers contain a cobalamin dependent methylmalonyl CoA mutase activity contraindicates the common belief that cobalamins are not involved in plant metabolism.

D. Regulation of Enzyme Levels. In continuing efforts to understand the biochemical mechanisms involved in the regulation of specific enzyme degradation, changes in activities of various enzymes caused by nutritional constraints have been studied in E. coli, K. aerogenes, Saccharomyces cerevisiae, Candida utilis, and Tetrahymena pyriformis.

(a) Glutamine synthetases of E. coli and K. aerogenes. Gradient gel elechophoresis of extracts of E. coli grown in the presence of ^{32}Pi under nitrogen limiting conditions disclosed the presence of multiple bands of ^{32}P- labeled proteins, that were identified as partially dissociated and degraded forms of ^{32}P-AMP-adenylylated GS. Similar degradation products were produced by the incubation of exogeneous ^{13}C-AMP-adenylylated GS with cell free extracts of E. coli prepared by gentle lysis of lysozyme treated cells but not with extracts prepared by sonication. Whether or not the unique capacity of lysozyme prepared extracts to degrade GS is due to preservation of an endogeneous degrading enzyme system by the gentleness of the extraction procedure (as has been assumed by other workers who have used this procedure) or is an artifact dependent upon the very low intrinsic proteolytic activity of lysozyme itself, is under investigation.

Glutamine synthetase in nitrogen deficient resting cell suspensions of K. aerogenes is inactivated by an energy dependent mechanism that leads to a loss of immunoreactive GS protein. Cell-free extracts prepared by sonication also catalyze inactivation of GS but without loss of immunoreactive material. The extract dependent inactivation is dependent upon Fe^{3+}, either DPN or TPN, O_2, and a nondialyzable heat labile factor (enzyme ?) in the extract. This inactivation is stimulated by various adenine nucleoside di- and tri-phosphates. It is inhibited by antimycin A, anaerobiosis and sulfhydryl reactive reagents, but not by serine protease inhibitors. The possibility that this inactivation involves a cytochrome mediated oxidation which represents the first step in enzyme degradation is under investigation.

(b) Phosphofructokinase of tetrahymena pyriformis. The phosphofructokinase (PFK) of T. pyriformis is membrane bound. The PFK in membrane vesicles undergoes rapid inactivation in the presence of NaF, MgATP and a soluble heat labile, non-dialyzable factor (enzyme ?) present in the non-particulate cellfraction. ATP cannot be replaced with either adenosine, pyrophosphate, c-AMP, AMP or α-β-methylene analogs of ATP; however, the ATP analog imidodiphosphate, AMP-P-NH-P could replace ATP. The inactivation is not inhibited by a number of serine protease inhibitors nor does it occur with a soluble form of PFK obtained by treatment of vesicles with triton-X-100. Whether the ATP dependent inactivation is due to ligand induced conformational changes, subunit dissociation, or to covalent modification of PFK is under investigation.

(c) Glutamate dehydrogenase of yeast. Other studies have shown that the transfer of glutamate grown Candida utilis to starvation (no nitrogen) medium, or to medium containing NH_4^+, leads to a decrease in catalytic potential, and to a rapid shift, from pH 8.0 to pH 7.0, in the pH optimum of the NAD dependent glutamate dehydrogenase (NAD-GDH). Afterwards, addition of glutamate to the starvation medium leads to complete reversal of the observed changes, that is independnet of protein synthesis. The pH 8.0 enzyme has been purified to homogeniety and antibodies raised against it. These antibodies cross react with both enzyme forms, and will be used for the

selective precipitation of the (DPN-GDH) before and after treatment of the cells with ^{32}Pi, to determine if the changes in enzyme activity involves a covalent modification by either phosphorylation or nucleotidylation.

During glucose starvation the TPN-dependent glutamate dehydrogenase (TPN-GDH), but not the DPN-GDH of Saccharomyces cerevisiae undergoes a rapid inactivation which can be inhibited by uncouplers of oxidative phosphorylation inhibitors of protein synthesis and the protease inhibitor, phenylmethylsulfonyl fluoride. Because inactivation of TPN-GDH does not occur in cell free extracts, efforts are being made to determine if inactivation will occur in permeabilized cells or in protoplasts.

Regulation of microtuble assembly. Sodium fluoride inhibits the in vitro GTP dependent assembly of microtubules in crude supernatants but not in purified tubulin preparations. The fluoride effect is partly explained by the demonstration that fluoride leads to the accumulation of GMP which is an inhibitor of polymerization; as are also AMP and CMP. It is well know that specific binding of colchicine to tubulin prevents the assembly and causes disassembly of microtubules. By means of fluorometric techniques and the use of radioactive colchicine it was established that colchicine binding is a two step process consisting of an initial rapid equilibrium reaction ($K_2 = 3.8 \times 10^{3} M^{-1}$) followed by a rate limiting protein conformational change ($K_2 \cong 2.8 \times 10^{-2} sec^{-1}$). Furthermore, tubulin is modified by a component released from brain particulate fractions by gentle sonication and detergents, which causes a 3-5 fold decrease in the rate of dissociation of the colchicine-tubulin complex without denaturation of the tubulin and no apparent change in the rate of binding.

Annual Report of the
Section on Intermediary Metabolism
and Bioenergetics
Laboratory of Biochemistry
National Heart, Lung and Blood Institute
July 1, 1976 through September 30, 1977

The research activities of the investigators in the Section on Intermediary Metabolism and Bioenergetics have been concerned with (1) the anaerobic metabolism of certain amino acids and nicotinic acid with particular reference to the characterization of the components of the selenium dependent glycine reductase system, the pyruvate-dependent proline reductase and B_{12}-coenzyme-dependent α-methylene glutarate mutase reaction (an intermediate step in the fermentation of nicotinic acid by Clostridium barkeri) and (2) The metabolism of formate and other one-carbon compounds by methane-producing bacteria and characterization of the selenium and molybdenum dependent formate dehydrogenase of Methanococcus vannielii.

Selenium Biochemistry and Anaerobic Oxido-Reduction Reactions:

Two of the three presently known selenoenzymes are the subject of studies in the Section on Intermediary Metabolism and Bioenergetics. These enzymes, glycine reductase and formate dehydrogenase, catalyze oxidation-reduction reactions that are important in the over-all energy-yielding processes of many anaerobic bacteria. Clostridium sticklandii, which synthesizes both glycine reductase and formate dehydrogenase, and Methanococcus vannielii, which produces high levels of formate dehydrogenase, serve as sources of experimental material. Glycine reductase and the formate dehydrogenase of these organisms are complexes of dissimilar polypeptides. In its reduced form the selenoprotein subunit of glycine reductase is a 12,000 dalton, acidic heat stable protein that contains a single selenocysteine residue and two cysteine residues. The formate dehydrogenase (M. vannielii) selenoprotein subunit is considerably larger; its selenium moiety is tentatively identified also as a selenocysteine residue.

Formate dehydrogenase of M. vannielii exists in two different molecular forms and the proportion of these forms varies with growth conditions. One of these, molecular weight about 200,000, is the predominant species when the bacteria are cultured in selenium deficient media. This form contains 2 equivalents of molybdenum and about 15 of iron per 200,000 daltons but no selenium. A larger molecular weight species ($\geq$ 500,000 daltons) that contains ^{75}Se is produced when 1 μM ^{75}Se-selenite is added to the culture medium. Fe and Mo are also present in this form. If tungsten and selenite are both added as supplements, then the larger molecular weight formate dehydrogenase species is produced almost exclusively and if labeled tungstate is used, ^{185}W is found in the purified enzyme. The larger molecular weight enzyme can be dissociated into a selenoprotein component plus an Fe-Mo species similar in size and properties to the 200,000 dalton form. It appears that partial replacement of tungsten for molybdenum may serve to stabilize the larger molecular weight selenoprotein form of the enzyme. Since growth of M. vannielii is stimulated by tungstate, the tungsto enzyme may be the more active species. Both forms

of formate dehydrogenase reduce tetrazolium dyes and are maximally active in this assay at pH 9 and 50-60°C. In the presence of an additional protein and its readily dissociable cofactor ($Factor_{420}$), an unidentified fluorescent electron carrier present in the methane bacteria, the 200,000 molecular weight formate dehydrogenase catalyzes the reduction of NADP. Thus a formate dehydrogenase-NADP-oxido-reductase is reconstituted from these proteins. Reconstitution studies with the larger selenium containing formate dehydrogenase are in progress.

The individual components of the glycine reductase complex of C. sticklandii have been further purified and characterized. Protein B, which is very sensitive to carbonyl reagents, catalyzes a slow exchange of tritium between water and the methylene carbon of glycine. This exchange, presumably via a Schiff base intermediate, serves as an assay for the protein during purification. Glycine reductase reconstituted from homogeneous preparations of protein A (the selenoprotein) and protein B plus highly purified fraction C requires both orthophosphate and ADP in order to convert glycine to acetate and ammonia with dithiothreitol as reducing agent. Since these enzymes are freed of adenylate kinase, the system now is completely dependent on ADP and AMP can no longer be utilized as acceptor. All three enzyme fractions and all reactants are required for NH_3 liberation and also for ADP conversion to ATP; no partial reactions have been detected. The initially determined stoichiometry of the process

$$1 \text{ glycine} + 1\ R(SH)_2 + 1\ Pi + 1\ ADP \rightarrow 1 \text{ acetate} + 1\ NH_3 + 1\ R\begin{matrix}S\\S\end{matrix} + 1\ ATP$$

is observed with the reconstituted system and this confirms the earlier conclusion that phosphate esterification and ATP synthesis are an integral part of the overall reaction. In preliminary experiments with labeled phosphate and labeled ATP, reaction conditions and isolation procedures have been developed that should allow us to determine if the phosphate ester intermediate in the process could be either a thiophosphate or a selenophosphate derivative of the selenoprotein. Either the highly reactive selenocysteine or cysteine residues of this protein are attractive candidates for such a role.

The glycine reductase selenoprotein gives a positive test for carbohydrate (periodate-basic fuchsin Schiff reagent) and both neutral and basic reducing sugars are detected in acid hydrolysates. Identification of the protein as a glycoprotein probably explains the blocked amino terminus. ^{75}Se-labeled protein synthesized in the presence of antibiotics that block glycosylation reactions exhibits lower than normal glycine reductase activity and also decreased antigenic activity suggesting that the glycosyl groups are important for interaction with the other proteins of the glycine reductase complex. Growth experiments with radioactive sugars to label the glycosyl groups are in progress and this should facilitate identification.

Studies on the mode of biosynthesis of the glycine reductase selenocysteine residue and also of formate dehydrogenase of C. sticklandii show that selenium incorporation from labeled selenite into both is prevented by antibiotics that inhibit either messenger RNA synthesis or protein synthesis. Although selenocysteine is utilized more efficiently than selenite, formation of biologically active glycine reductase selenoprotein from the added selen

amino acid also requires active protein synthesis. A semi-*in vitro* system has been developed to further investigate the mechanism of the highly specific incorporation of selenium into the bacterial selenoproteins that require this trace element for their activity.

Proline Reductase:

Following purification and characterization of the proline reductase of *Clostridium sticklandii* which proved to be a sulfhydryl protein consisting of 10 similar subunits each terminating with a pyruvate residue, studies were carried out to identify the electron carriers required to link the reductase with NADH, the normal electron donor. A copper containing protein which can be reduced by dithionite and then in turn reduces proline reductase has been purified to homogeneity. This is an 86,000 dalton protein comprised of two 43,000 dalton subunits and approximately one gram atom of copper. The following proline reducing systems are now defined:

(1) dithiothreitol plus proline reductase and proline
(2) dithionite plus copper protein plus proline reductase and proline,

and a third system (3) consisting of at least one additional protein, possibly a flavoprotein, and the copper protein that links proline reductase to NADH, the biological electron donor. Studies are in progress to further characterize these proteins, to establish their mode of action and to determine if the copper in the copper protein changes valency during the reaction. If so, this would be an example of an unusual copper protein that transfers electrons anaerobically rather than reacting with molecular oxygen.

Mechanistic studies on B_{12}-coenzyme dependent reactions:

The B_{12}-coenzyme dependent enzymes, α-methylene glutarate mutase of *Clostridium barkeri* and D-α-lysine mutase of *Clostridium sticklandii* catalyze two different types of rearrangement reactions. Conversion of α-methylene glutarate to methylitaconate involves cleavage of a carbon-carbon bond and the migration of an acrylyl moiety. Cleavage of a carbon-nitrogen bond and migration of the amino group to an adjacent carbon occurs when D-α-lysine is converted to 2,5-diaminohexanoate. Specifically ^{3}H labeled substrates for these enzymes have been prepared by organic syntheses and enzymes freed of competing side reactions have been isolated in order to gain further insight into the reaction mechanism of B_{12} coenzyme catalyzed reactions.

Annual Report of the
Section on Protein Chemistry
Laboratory of Biochemistry
National Heart, Lung and Blood Institute
July 1, 1976 through Sept. 30, 1977

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 protein-protein interactions in enzyme catalysis and regulation.

Glutamine synthetase, a strictly regulated enzyme in E. coli, is a dodecamer with twelve catalytic sites. Interactions of divalent cations, substrates, and inhibitors with glutamine synthetase from E. coli have been studied by microcalorimetry, equilibrium dialysis, pH, spectral, and kinetic techniques. Calorimetrically measured heats have provided information on the separateness of ligand binding sites and on proton uptake or release in binding reactions. Thermal saturation curves for the binding of low-affinity ligands to the enzyme have given thermodynamic binding parameters. Thermodynamic parameters for the enzyme reacting with substrates, ADP, L-glutamine, and L-glutamate, and inhibitors, L-alanine, AMP, and L-tryptophan, have been obtained for various combinations of ligands. In forming the ternary catalytic complex ADP-MnGS-Gln, an ordering effect of L-glutamine and a disordering effect of ADP were observed. In contrast to the positive entropy of binding ADP to the Mn-enzyme, binding of the feedback inhibitor AMP has $\Delta S' = -20$ cal/deg·mole subunit. In addition, a substantial proton uptake occurs upon the binding of AMP, but not of ADP, to the enzyme. Other calorimetric results on L-glutamate and L-alanine binding to the Mg-enzyme indicate that there is a synergistic effect between the binding of L-alanine and ADP + P_i and an antagonistic effect between L-glutamate and ADP + P_i whereas ATP is synergistic to the binding of both L-alanine and L-glutamate.

Equilibrium and kinetic measurements of glutamine synthetase-metal ion interactions have been made. Equilibrium data of Mn^{2+} binding to unadenylylated, partially adenylylated and fully adenylylated enzymes suggest that heterologous interactions between unadenylylated and adenylylated subunits in hybrid enzyme molecules (i.e., those containing both types of subunits) decrease the apparent affinity of n_1 subunit sites for Mn^{2+}. The substrate L-glutamine has the capacity to increase by about 30-fold the apparent association constant for Mn^{2+} binding to n_2 subunit sites without affecting Mn^{2+} binding to the high-affinity n_1 subunit sites; the influence of L-glutamine on enhancing the affinity of the lower affinity n_2 subunit sites for Mn^{2+} is proportional to the binding of L-glutamine to catalytic sites. Studies of a reversible thermal transition of glutamine synthetase have suggested that occupancy of both the n_1 and n_2 divalent cation sites of each subunit is important in the stabilization of the protein structure. Information on the mechanism of metal ion interactions with the enzyme is being obtained by studies employing dye-metal ion complexes and pH-indicator dyes.

A specific labeling of the catalytic subunit site of unadenylylated glutamine synthetase by pyridoxylation at phosphate binding sites has been achieved. The covalently attached pyridoxamine phosphate groups act as spectral probes for ligand interactions with the enzyme. Studies with this derivative will give information on the topography of divalent cation and other ligand binding sites.

In a separate study, polymerization reactions of γ-G immunoglobulins are being tested in order to produce specific agglutinating activities.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00201-06 LB

PERIOD COVERED
July 1, 1976 - September 30, 1977

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

J. M. Poston, Ph.D. Section on Enzymes LB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.0	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)

In the study of the branched-chain amino acids, the initial step in the catabolism of leucine is catalyzed by leucine 2,3-aminomutase. The product, β-leucine, is deaminated and cleaved to give acetate and isobutyrate. Leucine 2,3-aminomutase is dependent upon coenzyme B_{12} and is stimulated by FAD, coenzyme A, DPN, and pyridoxal phosphate. The enzyme is found, in addition to bacteria and mammals, in bean seedlings, ryegrass, spinach, and potato tubers.

Project Description

Objectives: The catabolism of the branched-chain, amino acids, leucine, isoleucine, and valine remains incompltely understood even though a pathway of catabolism has been outlined in bovine and rat liver. Certain inborn errors of metabolism, e.g., maple syrup urine disease and isovaleric acidemia, have been described which implicate faulty metabolism of these amino acids. The branched-chain amino acids have been shown to participate in the Strickland reaction and until recently, the only fermentation of these amino acids has been in Stickland pairs. Now, however, several strains of organisms have been isolated which grow on leucine in a single amino acid fermentation. The objectives of this project are to establish the fermentation pathways of leucine and the other branched-chain amino acids in these organisms, to examine the enzymes responsible for the various metabolic steps in these fermentations, and to explore the distribution of these pathways in other species.

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

α-leucine → β-leucine → B-ketoisocaproate → acetate + isobutyrate

The first step in this pathway is catalyzed by the enzyme, leucine 2,3-aminomutase, which requires coenzyme B_{12} [adenosylcob(III)alamin]. In assays in which β-leucine is the substrate, production of α-leucine is stimulated by the addition of the cofactors FAD, coenzyme A, DPN, and pyridoxal phosphate. It is not yet clear what part of these cofactors play in the enzymic activity.

Enzyme activity has been partially purified from both Clostridium sporogenes and Clostridium lentoputrescens although enzyme stability continues to hamper purification attempts.

In addition to the finding of leucine 2,3-aminomutase activity in several clostridia, in liver (human, rat, sheep, African green monkey, and Rhesus monkey), in rat kidney, and in human leukocytes as reported previously, a collection of sera have been examined for the presence of β-leucine. This amino acid has been shown to be present in normal human sera at the concentration of 0.0635 ± 0.0412 mg per 100 ml (n=37). In a set of plasmas from England provided by Dr. S. Harvey Mudd of the National Institute of Mental Health, patients with pernicious anemia were found to have levels of β-leucine that appear to be elevated (i.e., greater than the normal sera by more than twice the standard deviation). Those patients that had been treated (presumably with vitamin B_{12}) had β-leucine within normal limits. Access to patients who have not been treated is extremely difficult and it will take some time before a sufficiently large series of of sera have been examined to be able to draw conclusions from such findings.

The surprising discovery that freshly harvested etiolated bean seedlings possess leucine 2,3-aminomutase activity reported last year has been extended by unequivocal evidence that the cobalamin-dependent activity is present as a part of the bean seedling itself rather than being an activity present because of bacterial contamination. Such cobalamin-dependent activity has also been found in ryegrass seedlings, spinach-leaf acetone powder, and in potato tubers. These four plants represent types of such diverse nature that it must be assumed that B_{12} material may, in fact, be found quite generally in the plant kingdom.

Extracts of potato tubers have also been found to contain methylmalonyl-CoA mutase activity. This activity, also cobalamin dependent, has not previously been shown to be present in plant tissue and its presence in potatoes demonstrates that the cobalamin dependence is not limited to a single enzyme in plants.

Proposed Course of Action: The leucine mutase will be purified and characterized. To this end, the conditions which yield maximum activity in cell cultures will be established and sources which may yield stable enzyme will be examined. The nature of the B_{12} involvement will be established and the nature of the other cofactor involvement will be examined. The distribution of the pathway in normal and diseased mammalian and human tissues will be studied as will its relation to plant development. The β-leucine deaminase (or transaminase) will be examined and the fate of the nitrogen will be determined. The β-ketoisocaproate cleavage enzyme will be examined and its cofactors established.

Relevance to Biomedical Research: This study impinges on at least three areas of medical concern, 1) the mode of action of vitamin B_{12} in its metabolic roles, 2) the means by which organisms catabolize food materials, and 3) the probable nutritional value of plant material with regard to B_{12} material. The second area is directly concerned with several inborn errors of metabolism that have been shown to be devastating to the well-being of humans especially in the instances of maple syrup urine disease, isovaleric acidemia, and disorders of the catabolism of short-chain acids. 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.

Publications:

Poston, J. M.: Leucine 2,3-aminomutase: A colbalamin-dependent enzyme present in bean seedlings. Science, 195, No. 4275, 301-302, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00202-06 LB

PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)
Kinetics, Regulation and Mechanisms of Biochemical Reactions

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

PI:	P. B. Chock	Section on Enzymes	LB NHLBI
	Sue Goo Rhee	Staff Fellow	LB NHLBI
	Judith R. Bale	Staff Fellow	LB NHLBI
	Charles Y. Huang	Scientific Consultant	LB NHLBI
	E. R. Stadtman	Chief, Laboratory of Biochemistry	LB NHLBI
OTHER:	Joseph J. Villafranca	IPA	
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	Emily Noiman	(June 1 - July 15)	

COOPERATING UNITS (if any) M. Greifner, Biomedical Engineering & Instrumentation Branch Division of Research Service, NIH; Sydney Kustu, University of California, Davis. T. R. Royt, Optical Science Division, O.T.B., Naval Research Laboratory; Forrest Foor, MIT

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SSection on Enzymes

INSTITUTE AND LOCATION
NHLBI,NIH, Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.1	3.7	.4

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)

(1) Analysis of cyclic cascade system shows that it is a superior method for metabolic regulation of key enzymes. It provides; (a) A well regulated signal amplification which is necessary for hormonal induced responses and neurol transmission. (b) A flexible regulatory system since a large number of allosteric effectors can interact with various proteins in the cascade to vary its enzymic activity; (c) A more sensitive response with respect to the change in effector concentration by allowing a given effector to interact with more than one protein in the cascade; (d) A dynamic process for regulation instead of ON-OFF switch mechanism. All these properties have been confirmed experimentally for the monocyclic system with the adenylylation and deadenylylation of glutamine synthetase system. (2) Spatial relationship between adenylyaltion site and the catalytic site of glutamine synthetase was established. In addition, the existence of allosteric sites for feedback inhibitors has been reaffirmed. (3) The interrelationship between effectors and substrates in the adenylylation-deadenylylation of glutamine synthetase was established. (4) Other work includes mechanistic study of alkaline phosphatase, genetic study of UTase and UR enzymes, and the development of laser heating T-jump machine.

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Project Description:

Objectives: (1) To set up a laboratory for the study of fast kinetics of reactions, particularly for studying individual steps of enzymic reactions and protein-ligand interactions. (2) With this fast kinetic technique and other physical and chemical methods, to elucidate the biochemical action of glutamine synthetase and glutamate synthase from Escherichia coli. (3) Theoretical analysis of cyclic cascade system in the regulation of key enzymes in metabolism and its possible role as a well regulated amplifier for neuro signal transmission. (4) Isolation of the regulatory proteins in relatively large quantity to allow detail mechanistic study and experimentally verify the validity of the cyclic cascade system described in (3). (5) To study the kinetics and mechanism of DNA-repressor interaction utilizing the fluorescence technique. (6) To further explore the role of covalent modification in metabolic regulation.

Major Developments:

With the help of Dr. T. R. Royt from the Naval Research Laboratory, we improved the prospect significantly in terms of converting an old neodynium-glass laser into a heat source for the laser heating temperature jump machine. The Q switching is achieved by using a home-built dye Q switching cell. A multi-passed method will be used to maximize the laser energy after Q switching. Since the 1.06 μ beams produced by the laser is virtually transparent for water, it is necessary to red shift this wavelength by stimulated Raman effect. For this purpose, a high pressure cell was built to maintain an 80 atm of H_2, or CH_4 or D_2. An experimental model sample cell was also built. With this sample cell, one can achieve a relatively even heating for the laser beam path and allow one to follow the reaction by either optical density or fluorescence change. When the building of this instrument is completed, one can follow reaction rates up to 50 nanosecond half-life under a relatively mild condition. The system will be used to study small molecule (such as ligand or drug)-protein interaction.

Major Findings:

(1) Theoretical analysis of interconvertible enzyme cascade in metabolic regulation. Unlike the unidirectional cascade which is involved in blood coagulation and complement fixation, the cyclic cascade, which involves in the covalent modification of interconvertible enzyme system is designed for controlled amplification of primary stimuli in the regulation of key enzymes in metabolism. A schematic representation of the cyclic cascade is shown in Fig. 1, where

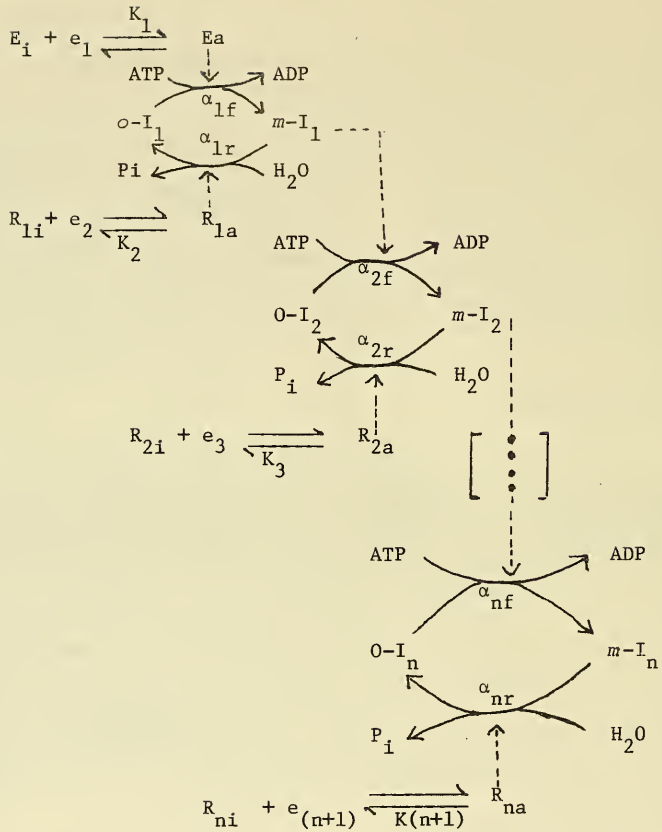


Figure 1

$o\text{-}I_n$ and $m\text{-}I_n$ are covalently unmodified and modified enzyme, respectively; α_{nf} and α_{nr} are defubed as $\alpha_{nf} = \frac{k_{nf}}{K_{nf}}$, $\alpha_{nr} = \frac{k_{nr}}{K_{nr}}$; k_{nf} and k_{nr} are specific rate constants for the forward and reverse reactions as designated. K_n is the dissociation constant for the designated equilibrium; K_{nf} and K_{nr} are dissociation constants for $o\text{-}I_n \cdot m\text{-}I_{(n-1)}$ and $m\text{-}I_n \cdot R_{na}$, respectively and n represents the number of cycles. With the assumption that (i) rapid equilibrium is maintained for the formation of enzyme-enzyme and enzyme-effector complexes, (ii) the concentrations of enzyme-enzyme complexes are negligibly low so that

$$[I_n] \simeq [o\text{-}I_n] + [m\text{-}I_n]$$

$$[E] \simeq [E_i] + [E_a]$$

$$[R_n] \simeq [R_{ni}] + [R_{na}]$$

where $[I_n]$, $[E]$ and $[R_n]$ are total concentration of I_n, E and R_n, respectively; and (iii) the concentrations of allosteric effectors, e_n are maintained at constant levels for a given metabolic state. Furthermore, the ATP dependency is ignored since the concentration of ATP in the cell is normally maintained at a constant level which is several orders of magnitude higher than the substrate enzymes. The results obtained from the equations derived with these assumptions are compatible with those obtained with a much more complex equation derived from a less restricted assumption.

With the assumptions described above, a general equation for calculating the fractional activity of I_n in cyclic cascade system containing n cycles is:

$$\frac{[m\text{-}I_n]}{[I_n]} =$$

$$\left[\frac{\alpha_{1r}\alpha_{2r}\alpha_{3r}\cdots\alpha_{nr}[R_1][R_2]\cdots[R_n][e_2][e_3]\cdots[e_{(n+1)}](K_1+[e_1])}{\alpha_{1f}\alpha_{2f}\alpha_{3f}\cdots\alpha_{nf}[E][I_1][I_2]\cdots[I_{(n-1)}][e_1]([K_2+[e_2])(K_3+[e_3])\cdots(K_{(n+1)}+[e_{(n+1)}])} \right.$$

$$+ \frac{\alpha_{2r}\alpha_{3r}\cdots\alpha_{nr}[R_2][R_3]\cdots[R_n][e_3][e_4]\cdots[e_{(n+1)}]}{\alpha_{2f}\alpha_{3f}\cdots\alpha_{nf}[I_1][I_2]\cdots[I_{(n-1)}](K_3+[e_3])(K_4+[e_4])\cdots(K_{(n+1)}+[e_{(n+1)}])} +$$

$$\left. \cdots + \frac{\alpha_{nr}[R_n][e_{(n+1)}]}{\alpha_{nf}[I_{(n-1)}](K_{(n+1)}+[e_{(n+1)}])} + 1 \right]^{-1} \qquad (1)$$

Fig. 1 and equation (1) show that covalent modification and demodification of interconvertible enzymes are dynamic processes by means of which the specific activity of the enzyme is varied by shifting the steady state distribution between active and inactive forms. The concept is more realistic and is certainly superior from the standpoint of regulation than earlier concepts in which interconvertible enzymes were considered to be "metabolic switches" which could be turned ON and OFF in response to allosteric stimuli. The latter concept implies that the respective converter enzymes that catalyze the modification and demodification reactions are activated and inactivated, reciprocally, in an "all or none" manner. With the dynamic mechanism, however, activity of an interconvertible enzyme can vary smoothly, over a wide range, in response to changes in allosteric stimuli, and will become stabilized at a fixed level commensurate with the metabolic state of the cell. However, under certain extreme conditions, cyclic cascades can assume the characteristics of unidirectional cascades. For example, in the absence of allosteric activator(s) or in the presence

of allosteric inhibitors(s) for the regeneration cascade regenerates $o\text{-}I_n$) would lead to an essentially irreversible forward cascade (generates $m\text{-}I_n$). Under this situation the cyclic cascade would function as a unidirectional cascade. Likewise, loss of forward cascade activity is also feasible, e.g. due to the complete removal of the allosteric effector, e_1, in which case the regeneration cascade would become unidirectional. As a consequence, temporal reciprocal losses of the forward cascade and regeneration cascade activities could lead to temporal alternation in the complete modification and demodification of the interconvertible enzyme, respectively; i.e., to an ON-OFF type of regulation.

A closer look at equation (1) shows that a minimum of three enzymes and five additional parameters are introduced for each additional interconvertible enzyme cycle. Since every enzyme in the cascade can be a separate target for one or more positive or negative metabolite effectors, such that by means of allosteric and substrate interactions with the cascade enzymes, the concentrations of a multitude of regulatory signals can be sensed and their effects integrated to determine the steady state distribution between active and inactive forms of the target enzyme, and hence its catalytic activity. In addition, multiple patterns of regulation can be achieved by varying the role of effectors in the activation or deactivation of the converter enzymes. Therefore a cyclic cascade system would provide a remarkable flexibility for metabolic regulation.

Unlike the unidirectional cascades, where signal amplification is uncontrollable and it leads to an explosive response to primary stimuli, equation (1) shows that the amplification capacity of cyclic cascade is susceptible to very fine regulation by responding to fluctuations in the concentrations of many different metabolites. The amplification is derived from the fact that the response of the target enzyme in a cascade to a primary stimulus, e_1, is a multiplicative function of various parameters in the cascade. Since the total number of these parameters is proportional to the number of cycles in the cascade, we have shown that a concerted effect of only two-fold variations in the contributions of each parameter can yield a 3×10^2, 10^5, 3×10^7 and 10^{10}-fold amplification of a primary stimulus in a one, two, three and four cycle cascade, respectively. Consequently, when several cascade parameters are altered simultaneously, interconvertible enzymes can respond to effector concentrations that are well below the dissociation constants of the effector-allosteric enzyme complexes.

In addition, the cyclic cascades are capable of generating a highly "cooperative-type" of response to the variation of a given effector concentration. Therefore, it increases the sensitivity in response to the change in allosteric effector. The rationale behind this property is derived from the fact that it is possible for a given effector to interact with more than one reaction step in the cascade. Experimentally, such phenomena have been observed for the regulation of E. coli glutamine synthetase and mammalian pyruvate dehydrogenase.

Needless to say that such an elegant cascade type of regulation does not

function, in response to the change in Gln (α-Kg) level. The properties observed here for the monocyclic cascade systems are in accord with those predicted by the analysis described in the previous section.

(3) Spatial relationship between the covalent adenylyl site and the active site of glutamine synthetase by NMR and fluorescence methods. The activity of glutamine synthetase from E. coli is regulated by covalent modification of a specific tyrosyl residue in each subunit. With the enriched 2-^{13}C-ATP (90% enriched) we adenylylated the enzyme to form 2-^{13}C-AMP-o-tyrosyl)$_{12}$-E•Mg. The ^{13}C NMR spectrum of this modified enzyme has a line width of 80 Hz, indicating the adenosine moiety is mobile and it likely exists on the surface of the enzyme. When the diamagnetic Mg is replaced by paramagnetic Mn^{2+}, the line width increased to 150 Hz with Mn^{2+} bound to the tight metal binding site (n_1) and to 230 Hz with Mn^{2+} bound to both n_1 site and the metal nucleotide catalytic site (n_2). From the paramagnetic contribution to the line width the distances from each Mn^{2+} site to the ^{13}C in AMP were calculated (see Fig. 2A). Addition of substrate, ADP, Pi and glutamate did not alter the distance relationship. The enzyme was also adenylylated with a fluorescent analog of ATP, 1,N^6-etheno-ATP. The fluorescence emission from the (1,N^6-etheno-AMP-o-tyrosyl)$_8$ E•Mg is quenched by enzyme bound Co^{2+}. A 75% and 20% quenching was observed due to Co^{2+} binding to n_1 and n_2 site, respectively. From the quenching efficiency, the distances between the fluorescent moiety and n_1 and n_2 site were estimated (see Fig. 2B). In addition, the ^{31}P-NMR spectrum of fully adenylylated glutamine synthetase was also studied. From the paramagnetic line broadening due to bound Mn^{2+} or bound Co^{2+}, the distances between the phosphorus of the adenylyl group to n_1 and n_2, respectively, were evaluated (Fig. 2A). To complete the spatial relationship, the distance between the n_1 and n_2 was obtained from the work of J.J. Villafranca et. al. (BBRC 75 464, 1977).

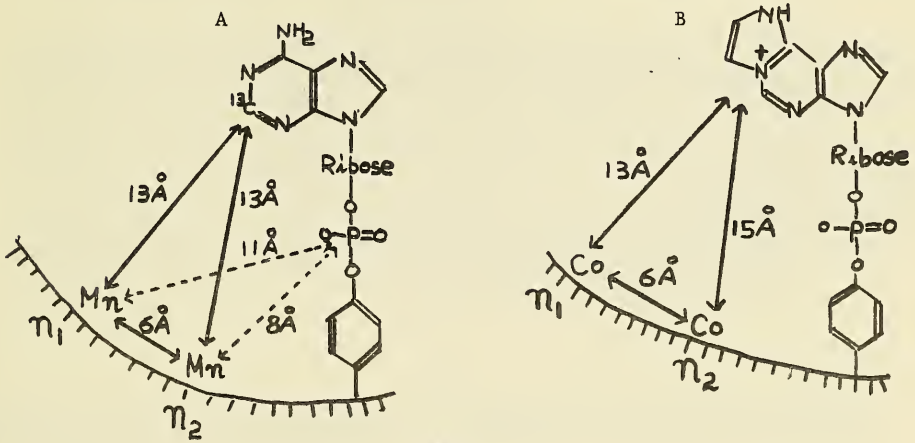


Fig. 2

(4) Mechanistic study of adenylylation and deadenylylation reactions for glutamine synthetase from E. coli. In the adenylylation reaction (equn. 3), Gln is an activator while α-Kg is an inhibitor. On the otehr hand,

$$12\ ATP + GS \xrightarrow[P_{IIA}]{ATase} GS(AMP)_{12} + 12\ PPi \qquad (3)$$

$$GS(AMP)_{12} + 12\ Pi \xrightarrow[P_{IID}]{ATase} GS + 12\ ADP \qquad (4)$$

Gln is an inhibitor while α-Kg is an activator for the deadenylylation reaction (eqn 4). The interrelationship in terms of binding affinity between the effectors and substrates was studied in pairs under the catalytic conditions. For example, results from the rate of adenylylation reaction with the concentration of glutamine being varied at different constant levels of P_{IIA} shows that the apparent K_m for Gln is 15, 6.7 and 2.2 mM with P_{IIA} = 0, 1.26 and 10.08 μm, respectively. In addition, P_{IIA} exhibits a strong synergistic effect on glutamine synthetase (substrate) binding to ATase (see Fig. 3); and Gln binding enhances ATP (substrate) binding. Since the K_m for Gln is reduced due to P_{IIA} binding, P_{IIA} indirectly enhances ATP binding. Experiments carried out in the absence of Gln show that the rate of adenylylation reaction in the presence of P_{IIA} is negligibly slow. Therefore the strong synergistic effect between P_{IIA} and Gln, P_{IIA} and glutamine synthetase, Gln and ATP, is responsible for expressing the role of P_{IIA} as an activator for the adenylylation reaction. The overall relationship observed is shown in Fig. 3 where s, a and ne represents synergistic, antagonistic and no effect, respectively, for a pair of ligands linked by a solid line.

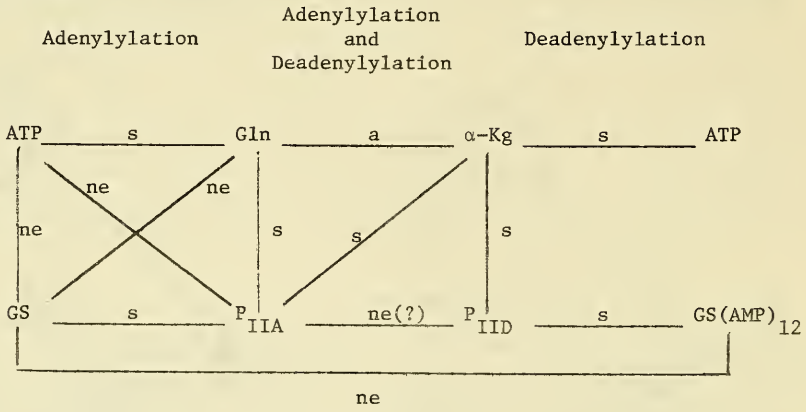


Fig. 3

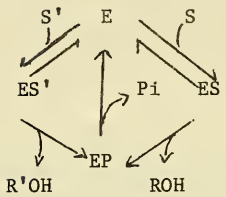
The binding of Gln and α-Kg exhibits an antagonistic effect but not mutually exclusive. Further, Fig. 3 shows that the binding of P_{IIA} and P_{IID} are not

competitive and the binding of adenylylated enzyme exerts no effect on the K_m for unadenylylated enzyme for the adenylylation reaction and vice versa for the unadenylylated reaction. These results suggest that ATase is likely a bifunctional enzyme in which a separate active center is utilized for the adenylylation and deadenylylation reaction. Such a hypothesis is also supported by the results of a mutation study with the ATase gene, glnE, in which glnE was altered such that the cell (Klebsiella aerogenes) lost the ability to adenylylate its glutamine synthetase, but maintained its deadenylylation activity in the presence of either P_{IIA} or P_{IID} (PNAS 72, 4844, 1975).

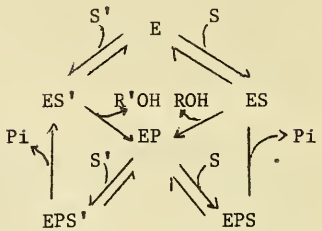
(5) Genetic study of UTase and UR enzymes. The regulatory protein, P_{II}, for the adenylylation-deadenylylation of glutamine synthetase is itself modulated by another covalent modification reaction, namely, uridylylation and deuridylylation of a tyrosyl residue in the P_{II} protein. The uridylylation of P_{II} is catalyzed by the UTase to form $P_{II}(UMP)_4(P_{IID})$, and the covalently bound UMP is removed enzymatically by UR. The partially purified UTase and UR show that both enzymic activities are copurified through several purification steps (JBC 250, 6264, (1975)). However, on storage, UR activity decreases much more rapidly than the UTase activity. The genetic study on the UTase gene, glnD, is designed to demonstrate whether these two enzymic activities are originated from a single polypeptide. When twelve mutant strains of glnD, for which UTase activity is lacking, from Salmonella typhimurium (Sydney Kustu), E. coli (Forrest Foor), Klebsiella aerogenes (Forrest Foor) and Klebsiella pneumoniae (Forrest Foor) were assayed for their UTase and UR activity, it was found that both UTase and UR were inactive for all strains tested. When two of the glnD mutants from S. typhimurium were reverted, both UTase and UR activities were recovered. These results and the observation that UR is less stable in storage compared to UTase suggest that UTase and UR activities are derived from a bifunctional polypeptide.

(6) Mechanistic study of E. coli alkaline phosphatase. Alkaline phosphatase is an enzyme with two identical subunits. A flip-flop mechanism (see Fig. 4) has been proposed to satisfy the Michaelis-Menton kinetics as well as the strong negative cooperativity demonstrated by substrate binding and enzyme phosphorylation (Eur. J. Biochem. 20, 124, (1971)). This mechanism involves substrate binding and phosphorylation at the first site followed by substrate binding at the second site to facilitate dephosphorylation at the first site (i.e. the activities at the two sites are coupled and alternating). Steady state kinetics using alternative substrates allows differentiation of the flip-flop model from a preferred model where the second site plays no role in dephosphorylation at the first site.

Preferred Model



Flip-Flop Model



$$\frac{E_o}{v} = a + b\left(\frac{1}{S}\right) + c\left(\frac{S'}{S}\right) \qquad (5)$$

$$= a + \alpha c + b\left(\frac{1}{S}\right) \qquad (6)$$

$$\frac{E_o}{v} = \frac{cS+dS'+eS^2+fSS'+gS'^2}{aS+bSS'} \qquad (7)$$

$$= \frac{c+\alpha d}{a+\alpha b}\left(\frac{1}{S}\right) + \frac{e+\alpha f+\alpha^2 g}{a+\alpha b} \qquad (8)$$

Fig. 4

The symbols used in Fig. 4 are: a, b, c, d, e, f and g and represent combinations of rate constants; S and S' are substrate and alternative substrate, respectively; α is the ratio of alternative substrate to substrate. Equation (7) suggests that in the presence of an alternative substrate, nonlinear steady state kinetics should be observed if the reaction proceeds via the flip-flop model (only in the presence of alternative substrate, the square terms do not cancel). Whereas equation (5), derived from the preferred model, predicts a linear 1/v vs 1/S plot which is in accord with the observed data when a fluorogenic compound, 6-bromo-2-hydroxy-3-naphthoyl-O-anisidine phosphate (NASBI-P), was used as substrate and CMP and AMP as alternative substrates. In all cases, the value for K_i agrees well with the K_m value when the alternative substrate was used as substrate. In fact, the K_m values so determined are significantly lower than those reported in the literature. This indicates that an inhibitor (likely Pi) was present in those reported experiments such that an apparent K_m was obtained. A second approach used to differentiate the two proposed models is a constant ratio of alternative substrate to substrate method developed by Huang (ABB (1977) in press). With this method equation (5) and (7) can be simplified to equation (6) and (8), respectively. Under this condition, the flip-flop model 1/v vs. 1/S plots at various α are linear and converging whereas the preferred model predicts that such plots are also linear but parallel. The experiments were carried out at pH 8.0 in 0.1 M Tris-HCl buffer, with AMP as substrate and NASBI-P and CMP as alternative substrate. The results are consistent with that predicted for the preferred model. It should be pointed out, however, that the method is not valid if the V_{max} values for both substrate and alternative substrate are the same.

The substrates were selected for their different V_{max}'s (above reference). Since the correct V_{max} is not easily determined due to strong Pi inhibition, the true V_{max}'s are being redetermined for computer simulation models.

In addition, since the flip-flop model is based on the fact that dephosphorylation step is facilitated by second substrate binding, the rate of dephosphorylation of ^{32}P-phosphorylated alkaline phosphatase was measured in the presence and absence of added substrate. In this experiment, a three-syringe-stopped-flow was used. The ^{32}P-phosphorylated enzyme was prepared at pH 5.5. The pH of this solution was rapidly jumped to pH 8.0 in the first mixing chamber with and without added substrate and the dephosphorylation was allowed to proceed for a given length of time (10-100 msec). The reaction was stopped by acid quenching in the second mixing chamber. The results show that the rate of dephosphorylation is not affected by the presence of substrates such as AMP, NASBI-P or Pi. Presently, the rate of phosphorylation is being measured with the stopped-flow method.

(7) Evidence for the existence of allosteric site(s) for amino acid feedback inhibitors on unadenylylated glutamine synthetase. In 1967 Woolfolk and Stadtman (ABB 118, 736, (1967)) reported that glutamine synthetase from E. coli is susceptible to eight nitrogen containing compounds of biological importance. Many of these compounds exhibit only partial inhibition when they are tested individually and their inhibition effects are cumulative when they are used collectively. Based on this observation and on the fact that some of these feedback inhibitors, e.g. L-Ala, Gly, show a noncompetitive inhibition pattern with respect to L-Glu, they proposed the existence of allosteric binding sites for the feedback inhibitors. However, a different hypothesis has been proposed by Dahlquist and Purich (Biochem. 14, 1980 (1975)) for which they suggested that the eight feedback inhibitors reported by Woolfolk and Stadtman simply bind to the substrate binding sites of the enzyme. Their argument is based on the fact that (a) enzyme-bound Mn^{2+} broadened the NMR spectra lf L-Ala methyl doublet and addition of L-Glu (up to 5 mM) to a solution containing L-Ala, enzyme and Mn^{2+} (in the absence of nucleotide) sharpened the L-Ala resonance; (b) L-Ala, like L-Glu exhibits a synergistic effect with ATP or ADP binding and (c) misquotation of a non-competitive inhibition (mixed) pattern by L-Ala with respect to L-Glu as a competitive pattern. In order to resolve the discrepancy described above, feedback inhibitors have been studied utilizing the fast reaction technique and an NMR method. With the home-built stopped-flow fluorometer, we can measure a small change in fluorescence intensity due to ligand binding. This method is particularly useful for measuring relatively weak binding constants, such as L-Glu, L-Ala, D-Val and Gly binding to $E\underline{\quad}_{1.0}$ Mg, when the addition of a realtively large volume of ligand solution necessary with a normal titration technique. The results of the dissociation constants so determined are:

$E\overline{\overline{}}_{1.0}Mg$ + Glu	17 ± 4 mM
$E\overline{\overline{}}_{1.0}Mg$ + L-Ala	> 60 mM
$E\overline{\overline{}}_{1.0}Mg$ + D-Val	> 50 mM
$E\overline{\overline{}}_{1.0}MgATP$ + L-Glu	4 ± 2 mM
$E\overline{\overline{}}_{1.0}MgATP$ + L-Glu	2 mM
$E\overline{\overline{}}_{1.0}MgATP$ + L-Ala	29 mM
$E\overline{\overline{}}_{1.0}MgATP$ + D-Val	9 mM
$E\overline{\overline{}}_{1.0}MgATP$ + Gly	25 mM
$E\overline{\overline{}}_{1.0}MgATP\ NH_3$ + L-Ala	20 mM
$E\overline{\overline{}}_{1.0}MgATP\ NH_3$ + D-Val	9 mM

for the following experimental conditions: pH 7.0, 15° in 50 mM HEPES-KOH, 20 mM $MgCl_2$ and 100 mM KCl; concentration of ATP, ADP and NH_3 when added are 4 mM, 1 mM and 20 mM, respectively. A K_d value of 24 mM was obtained previously for L-Glu from $E\overline{\overline{}}_{1.0}Mg{\cdot}ADP{\cdot}Pi{\cdot}L\text{-}Glu$ ([Pi] = 5 mM) at pH 7.6, 25°. The dissociation constants given above show a strong synergistic effect is observed between ATP or ADP and amino acid such as L-Glu, L-Ala, Gly and D-Val for binding to $E\overline{\overline{}}_{1.0}Mg$ at pH 7.0. The enhancement factor for L-Glu binding is 4 and ~8 in the presence of ADP and ATP, respectively. This is because with ATP presence, reaction intermediates (I_1 and I_2) for the biosynthesis are formed and interestingly, the ratio of I_2 to I_1 is 2.3 (PNAS 73, 476 (1976)). If ADP and Pi are present simultaneously, one observed antagonistic effects for L-Glu, but not for L-Ala. This is likely due to some degree of overlapping of oxygen binding sites for the extra oxygen atom in the ADP-Pi system compared to the ATP system and one of the γ-carboxyl oxygen atoms of L-Glu. In the case of L-Ala, it possesses no γ-carboxyl group, therefore no such overlapping is possible. The similarity in thermodynamic relationship between L-Glu and the amino acid inhibitors in the presence of ATP or ADP and the dissimilarity between L-Glu and L-Ala in the ADP-Pi system does not allow one to conclude whether all amino acids described here share a common binding site.

The observed polarity and the relative fluorescence amplitude changes due to the binding of amino acid(s) to either $E\overline{\overline{}}_{1.0}Mg$ or $E\overline{\overline{}}_{1.0}Mg{\cdot}ATP$ as compared to the calculated values based on the competitive binding hypothesis are given below:

Amino Acid Added (mM)	Observed ΔF (mV)	Expected ΔF for Competitive Binding (mV)
E + L-Glu (100)	410 ↓	
E + L-Ala (100)	360 ↓	
E + D-Val (100)	320 ↓	
E•L-Glu(100) + L-Ala(100)	80 ↑	> 35 ↓
E•L-Glu(100) + D-Val(100)	260 ↑	> 16 ↓
D•L-Ala(100) + D-Val(100)	380 ↑	> 50 ↓
E•ATP + L-Ala(150)	470 ↑	
E•ATP + D-Ala(100)	810 ↑	
E•ATP + Gly(100)	650 ↑	
E•ATP + D-Val(100)	800 ↑	
E•ATP•L-Ala(150) + Gly(100)	300 ↑	155 ↑
E•ATP•L-Ala(150) + D-Ala(100)	400 ↑	
E•ATP•L-Ala(150) + D-Val(100)	450 ↑	255 ↑
E•ATP•L-Ala(150) + L-Ala(100)	60 ↑	
E•ATP + L-Ala(100), Gly(100)	800 ↑	611 ↑
E•ATP + L-Ala(100), D-Ala(100)	920 ↑	
E•ATP + L-Ala(100), D-Val(100)	920 ↑	744 ↑
E•ATP•D-Val(100) + D-Ala(100)	30 ↑	
E•ATP•Gly(170) + L-Ala(170)	120 ↑	60 ↓
E•ATP•Gly(170) + Gly(170)	0	

These results show that the observed fluorescence amplitude either by simultaneous addition or by sequential addition of 2 different amino acids is consistently higher than the calculated value. In some cases, an opposite polarity is observed. These data indicate that L-Glu, L-Ala and D-Val bind at a separate site on the enzyme subunit and binding of one amino acid will induce a protein conformational change such that different fluorescence amplitude will be observed when a given amino acid is titrated into $E\underline{\quad}{}^{Mg}_{1.0}$ (or $E\underline{\quad}{}^{Mg}_{1.0}$•ATP) in the presence or absence of another amino acid. However, this argument is permissible only if there is no subunit interaction within the dodecamer in a manner that a L-Glu bound subunit when adjacent to a L-Ala bound subunit would vary the quantum yield of either L-Glu bound subunit or L-Ala bound subunit or both. Since to date there is no real evidence for the subunit interaction in glutamine synthetase, this assumption is probably justified. It should be pointed out that these results

provide no information on antagonist effects between two amino acids. In addition these data indicate that with the exception of D-Ala and D-Val, all amino acids can bind simultaneously on the enzyme subunit, and the binding of these amino acids will induce a protein conformational change.

The paramagnetic contribution to the $1/T_{1p}$ and $1/T_{2p}$ relaxation rates of the proton of L-Glu, L-Ala, Gly and D-Val were monitored at 220 MHz in the presence of $E\underline{\quad}(Mn)_2 \cdot ADP \cdot Pi$. The $1/T_{1p}$ values for the protons of these amino acids were also determined at 100 MHz. This frequency dependent study of $1/T_{1p}$ values permits a calculation of the correlation time, τ_c. With a known τ_c, the distances between Mn^{2+} and each set of protons was computed. The results show that the distance from the α-CH, β-CH_2 and γ-CH_2 of L-Glu to the nearest Mn is 7.8, 7.6, 6.9 Å, respectively, the distance for the α-CH and β-CH_3 of L-Ala to the nearest Mn is 9.2 and 9.6 Å respectively; the distance for α-CH of Gly to the nearest Mn is 8.7 Å; the distance for α-CH, β-CH and α-CH_3 of D-Val to the nearest Mn is 11, 10.6, 10.3 Å, respectively. In addition, the $1/T_{1m}$ for the protons of a given amino acid was monitored while a second aminod acid was being titrated into the solution. The data shows that D-Val and L-Ala; L-Glu and L-Ala; L-Glu and Gly, L-Ala and Gly can coexist on the enzyme surface. ^{13}C-NMR experiments were also carried out to show that AMP is located at least 20 Å away from the ADP Mn site. In conclusion, all results given in this section are in disagreement with the proposal that feedback inhibitors bind at a substrate site.

(8) Inhibition of glutamine synthetase by L-methionine-SR-sulfoximine (MSON). The irreversible inhibition of glutamine synthetase by MSON in the presence of ATP has been reported (JBC, 248, 3997, (1973)). The authors demonstrated that the inhibition is caused by the fact that MSON reacts with ATP on the enzyme to form tightly bound ADP and L-methionine-S-sulfoximine phosphate, an analog of the intermediate, α-glutamyl phosphate, for the biosynthetic reaction. However, based on the fact that MSON binds tighter to the enzyme than one of its substrates, L-Glu, a new explanation was proposed for the inhibition mechanism (JBC , 251, 7530 (1976)). The proposed explanation is that MSON mimicks the transition state, which is formed from NH_3 and glutamate. To resolve the discrepency, the mechanism of inhibition by MSON is investigated by both steady state kinetic and stopped flow method. The preliminary results showed when MSON was added to $E\underline{\quad}Mg \cdot ATP$, a relatively slow rise of tryptophan fluorescent intensity was observed. The half-time is larger than 40 sec. The half saturation point determined by this fluorescent change is 5 μM. On the other hand, the addition of MSON to $E\underline{\quad}Mg$ provoked a fast fluorescent intensity increase with half-saturation point at 2mM. Preliminary results show that this rate is MSON concentration dependent and a three step binding mechanism is required to explain the reaction time course. The steady state kinetic study shows that MSON is a competitive inhibitor with respect to glutamine in the transferase reaction and a Ki value of 20 μM was obtained.

Significance to Biomedical Research:

The object is to gain a better understanding of how enzymes function with respect to their catalytic and regulatory properties and to elucidate principle of interactions between effectors, regulators and proteins. The analysis of

the cyclic cascade model show that it plays a significant role in metabolic regulation of the key enzymes and it might be utilized in other biological processes in which signal amplification is important, such as neural transmission or hormonal induced responses.

Proposed Course of Research:

(1) To further expand the cyclic cascade model to substrate cascade and more complex enzyme cascade systems; and to 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. In this aspect, we plan to isolate UTase and UR enzymes; to study the protein-protein interaction between P_{II} protein and ATase, UTase, UR and P_{II}, ATase and glutamine synthetase by physical, chemical and immunological methods; to test the validity of the bicyclic cascade model and to study the role the effectors in this cascade system

(3) Kinetic and mechanistic study of glutamate synthase will be carried out. Both steady state and presteady state data will be used to elucidate the mechanism. In addition, the oxidation states of flavin and iron-sulfur cluster will be determined and attemps will be made to separate the two dissimilar subunits under mild conditions which preserve the iron-sulfur and flavin chromophores and allos reconstitution of active enzyme.

(4) To further explore the physical, chemical and immunological properties of unadenylylated and adenylylated glutamine synthetase. In particularly, we will utilize the fast reaction technique, NMR and ESR methods to elucidate the roles of effectors and to identify the intermediates in the catalytic cycle.

(5) To develop the laser heating temperature-jump machine and a slow heat exchange temperature-jump machine to cover the nanosecond and second range respectively.

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S. P. Chock, P. B. Chock and E. Eisenberg. Pre-steady State Kinetic Evidence for a Cyclic Interaction of Myosin Subfragment-1 with Actin During the Hydrolysis of ATP. Biochem. 15, 3244-3253, 1976.

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S. G. Rhee, P. B. Chock and E. R. Stadtman. Mechanistic Studies of Glutamine Synthetase from Escherichia coli: An Intergrated Mechanism for Biosynthesis, Transferase, ATPase Reactions. Biochim. 58, 35-49, 1976.

E. Garcia, S. Bancroft, S. G. Rhee and S. Kustu. The Product of a Newly Identified Gene, glyF, Is Required for Synthesis of Glutamine Synthetase in Salmonella. Proc. Nat. Acad. Sci. US, 74, 1662-1666, 1977.

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S. G. Rhee, R. Park, P. B. Chock and E. R. Stadtman. The Use of E. coli Glutamine Synthetase as a Model to Investigate the Allosteric Regulation of Monocyclic Interconvertible Enzyme Cascade Systems. Fed. Proc. 36, 777, 1977.

J. R. Bale and C. Y. Huang. Mechanisms of E. coli Alkaline Phosphatase: Kinetic Evidence Contrary to the Flip-Flop Model. Fed. Proc. 36, 635, 1977.

V. P. Werth and S. G. Rhee. Mechanistic Studies of the Unadenylylated Glutamine Synthetase from E.coli: Divalent Metal Ion Activation and Substrate Binding to Ca^{2+} Activated Enzyme. Fed. Proc. 36, 856, 1977.

P. B. Chock, S. G. Rhee and J. J. Villafranca. Regulation of Unadenylylated GS From E. coli: Direct Evidence for Separate Binding Sites for Substrates and Feedback Inhibitors. Fed. Proc. 36, 856, 1977.

J. J. Villafranca, S. G. Rhee and P. B. Chock. Spatial Relationship Between The Covalent Adenylyl Site and the Active Site of Glutamine Synthetase. Fed. Proc. 36, 856, 1977.

E. R. Stadtman and P. B. Chock. Superiority of Interconvertible Enzyme Cascades in Metabolic Regulation: Analysis of Monocyclic Systems. Proc. Nat. Acad. Sci. US, 74, 1977.

P. B. Chock and E. R. Stadtman. Superiority of Interconvertible Enzyme Cascades In Metabolic Regulation: Analysis of Multicycle Systems. Proc. Nat. Acad. Sci, US, 74, 1977.

E. R. Stadtman and P. B. Chock. Interconvertible Enzyme Cascades in Metabolic Regulation. in Current Topics in Cellular Regulation, Horecker and Stadtman (Ed)., Vol 13, 1977.

P. B. Chock, F. Eggers, M. Eigen and R. Winkler. Relaxation Studies on Complex Formation of Macrocyclic and Open Chain Antibodies with Monovalent Cations. Biophysical Chem. 6, 239-251 (1977)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00203-04 LB

PERIOD COVERED
July 1, 1976 - September 30, 1977

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

PI: Cynthia Oliver — Microbiologist LB NHLBI
OTHER: Earl R. Stadtman — Chief, Laboratory of Biochemisty LB NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.65	.1	.55

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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 investigation is to study the regulation of intracellular protein degradation in E. coli. Experiments previously undertaken in this laboratory using ^{3}H leucine labeling have indicated that E.coli stringent cells degrade protein more rapidly than isogenic relaxed cells under conditions of metabolic stress, particularly carbon or nitrogen starvation. Therefore, these conditions were selected for studies directed toward the isolation of one or more enzyme systems which exhibited a sharp reduction in intracellular level following provocation of the stringent response, as well as characterization of the cellular components responsible for this effect. Using ^{32}P as a probe to monitor changes in phospho-proteins an increase in low molecular weight labeled proteins was observed in extracts following a stringent response. Similar results were obtained by incubating ^{14}C-(AMP)-glutamine synthetase with cold extracts prepared in the same manner. Experiments have been designed to study these effects further. Techniques utilized in these studies have included polyacrylamide pore gradient electrophoresis, isotopic labeling, column chromatography, autoradiography, and enzymatic assay of functional proteins.

Project Description:

The control of intracellular enzyme level is important in metabolic regulation. Changes in these enzyme levels reflect either changes in the rate of protein synthesis at the transcriptional or translational level or changes in the rate of degradation. The metabolic control of the various aspects of protein synthesis has been well characterized but little is known about intracellular protein degradation or its regulation. The object of this investigation is to elucidate some of the basic cellular mechanisms underlying the process of protein degradation.

Major Findings:

I. Previous studies in this laboratory have led to the development of a pore gradient electrophoresis technique in tubes. However, because of difficulties in reproducing the gradient precisely either between or within batches of tubes a slab technique was developed and subsequently refined. It was found that by altering the concentration of the cross linking agent the molecular weight range of separation could be manipulated. Also, by changing the catalyst-accelerator ratio more stable polymerization properties could be obtained without generation of excess heat of polymerization. The slab gradients have several advantages over the tube gradients including, ease of handling, smaller cross sectional sample area allowing sharp resolution of much smaller total quantities of protein, shorter running time, and uniform gradients in which rare discontinuities can be easily visualized.

II. In a series of experiments in which cells were labeled with ^{32}P, an increase in low molecular weight material was observed (using gradient gels) in extracts from cells grown under stringent conditions. This effect was greatest with stringent, val S$^-$(ts) rel A+ cells compared with relaxed, val S-(ts) rel A- cells following temperature shift and concomitant nitrogen starvation. These phosphorylated peaks represented material ranging from 12,000 to 400,000 molecular weight. This material was sensitive to both trypsin and subtilisin. In addition, the phosphate moiety was neither dialyzable nor adsorbed on charcoal but sensitive to snake venom phosphodiesterase and insensitive to alkaline phosphatase, suggesting that this material was protein containing phosphate in covalent form. When labeled extracts were coelectrophoresed with cold excess glutamine synthetase (GS) as a marker, coincidence of the Coumassie blue bands and the phosphorylated peaks was observed. In addition, several smaller than 50,000 molecular weight species were obtained in labeled extracts but were not present in samples containing GS alone. Subsequently, it was demonstrated that under the conditions of electrophoresis employed in these experiments (low ionic strength, no Me++, pH 8.3) GS dissociated into single subunits and several multiple subunit forms. To test whether the smaller than GS subunit species (SS-GS) were derived from GS, unlabeled extracts were incubated with ^{14}C-(AMP)-GS and under these conditions labeled SS-GS species were obtained. These results suggested that these extracts had the capacity to generate labeled SS-GS from labeled adenylylated GS, presumably by proteolysis.

III. The generation of SS-GS species was observed only in extracts prepared with lysozyme but not in extracts prepared by sonic disruption. Previous studies in this laboratory had demonstrated that lysozyme binds and precipitates GS at low ionic strength. In the course of control studies with GS and lysozyme, it was found that lysozyme was capable of generating SS-GS species from GS but the profile obtained was quite different from that obtained with lysozyme prepared extracts. These results suggested that lysozyme preparations contained proteolytic activity either as a contaminant protein or as an inherent property of lysozyme, but that this activity was not solely responsible for the proteolysis observed in extracts. Subsequent experiments verified the presence of a non-specific esterase activity but only trace proteolytic activity was demonstrated using denatured hemoglobin, casein, albumin, azocasein, or azoalbumin as substrates. No hydrolysis of synthetic esters used in the assay of trypsin, chymotrypsin, carboxypeptidase A or B was demonstrated. A commercial supply house has verified trace proteolytic activity in all lysozyme preparations. Therefore, experiments were undertaken to separate the lytic and esterase activities by gel filtration under various conditions of pH and ionic strength but these experiments failed to resolve the two activities. One striking finding was that the ratio of the two activities was the same in lysozyme preparations from different commercial sources. However, heat denaturation studies, pH and ionic strength studies indicated that the activities behaved in a somewhat reciprocal manner. A survey of the literature revealed a study by Bruice (Bruice, Thomas C., Dennis Piszkiewicz, 1968, Biochemistry 7:3037.) in which he demonstrated an inherent esterase activity of lysozyme at a site distinct from the lysozyme lytic site. It has seemed plausible from his studies that the esterase site of lysozyme may be responsible for the trace proteolytic activity observed in these studies.

Proposed Course of Action:

The proposed course of action is twofold, (1) to obtain an esterase-protease-free lysozyme preparation and (2) to repeat the GS studies with extracts prepared with the modified and/or purified lysozyme preparation. Initial studies will be concerned with establishing the identity or non-identity of the sites involved in esterase and protease activity of lysozyme. In this regard a more sensitive assay or proteolysis is required and a fluorescent assay using phthaldehyde is being adapted for this purpose. If it is found that the esterase site is responsible for the trace proteolytic activity, the esterase site which contains the only histidine (his-15) in lysozyme could be selectively modified by carboxymethylation or photooxidation. Carboxymethylation (Bruice, Thomas C., Dennis Piszkiewicz, 1968, Biochemistry, 7:3037) has been shown to produce a lysozyme lacking esterase activity and retaining about fifty percent of the original lytic activity. If the esterase and protease activities are separate proteins or separate sites on lysozyme further effort will be directed toward separating the activities or additional site specific modification. Subsequent studies will be involved with using the purified and/or modified lysozyme preparation in studies similar to those described earlier with the hope of isolating and characterizing an intracellular protein degradation system.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 00204-10 LB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Protein Structure: Enzyme Action and Control

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

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OTHER:	Donald M. Powers, Staff Fellw (terminated 9/76)	LB	NHLBI
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COOPERATING UNITS (if any) D. A. Zopf, LBP, NIAMDD, NIH.
J.B. Hunt, Assoc. Prof. Dept. Chem., The Catholic Univ. of America, Washington, D.C. 20017 (2.5 mos., 1977; Title IV, Intergovernmental Personnel Act).

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SECTION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.56	3.26	.3

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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) Interactions of divalent cations, substrates, and inhibitors with glutamine synthetase from E. coli have been studied by microcalorimetry, equilibrium dialysis, pH, spectral, and kinetic techniques. A reversible thermal transition in the enzyme structure is also being studied. In addition, a specific labeling of the catalytic sites of the glutamine synthetase dodecamer has been achieved by reaction of this enzyme with pyridoxal phosphate, followed by borohydride reduction of the Schiff base. The pyridoxamine phosphate group acts as a spectral probe for the interactions of the modified enzyme with ligands.

(2) To produce a specific agglutinating activity, polymerization reactions of IgG antibodies are being studied.

PHS-6040
(Rev. 10-76)

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 conformation 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) Ultracentrifugation, calorimetric, and electrophoretic studies to determine macromolecular properties of biologically important proteins. (4) Kinetic studies of protein-metal ion interactions, using glutamine synthetase and dye-metal ion complexes and the enzyme + Me^{2+} in the presence of pH-indicator dyes. (5) To polymerize γG-immunoglobulin.

Major Findings:

(1) Equilibrium measurements of Mn^{2+} binding to glutamine synthetase of E. coli. (Investigators: D.M. Powers and A. Ginsburg). The fully adenylylated enzyme was found to have the same K_1^1 stability constant for Mn^{2+} binding to n_1 sites as that measured previously for unadenylylated glutamine synthetase. In contrast, partially adenylylated enzymes have lower K_1^1 values. This suggests that heterologous interactions between unadenylylated and adenylylated subunits in hybrid enzyme molecules (i.e., those containing both types of subunits) decrease the apparent affinity of n_1 subunit sites for Mn^{2+}.

The substrate L-glutamine has the capacity to increase by about 30-fold the apparent association constant for Mn^{2+} binding to n_2 subunit sites (K_2^1) without affecting Mn^{2+} binding to the high affinity n_1 subunit sites; with a saturating concentration of L-glutamine $K_2^1 \simeq K_1^1 = 1.9 \times 10^6 M^{-1}$ for Mn^{2+} binding to n_1 and n_2 subunit sites of the dodecamer. The influence of L-glutamine on enhancing the affinity of n_2 subunit sites for Mn^{2+} is proportional to the binding of L-glutamine to the enzyme subunits.

(2) Calorimetric studies on the interactions of substrates and inhibitors with glutamine synthetase from E. coli. (Investigators: A. Shrake and A. Ginsburg). Glutamine synthetase, a strictly regulated enzyme in E. coli, is a dodecamer with twelve catalytic sites. Heats of binding substrates and inhibitors to the enzyme have been measured at pH 7.1-7.2 and 30° in a batch-type microcalorimeter. Thermal saturation curves for the binding of low-affinity ligands to the enzyme have given thermodynamic parameters for different binding reactions. Heat measurements have provided information also on the separateness of binding sites and on proton uptake or release during ligand binding. Separate protein binding sites for the substrate L-glutamate and the feedback inhibitor L-alanine, and for the feedback inhibitors AMP and L-tryptophan have been demonstrated by calorimetric measurements with unadenylylated glutamine synthetase. Thermodynamic parameters for the following binding reactions have been obtained at 30° (standard state for hydrogen ion activity is $10^{-7.1}$–$10^{7.2}$M):

Reaction of GS Subunit*	ΔG' (kcal/mole subunit)	ΔH' (kcal/mole subunit)	ΔS' (cal/deg·mole)	Approx. H^+ Uptake
Mn_2GS + Gln	- 2.96	- 9.7	- 22	0
Mn_2GS + ADP	- 7.60	- 5.7	+ 6	0.1
ADP-Mn_2GS + Gln	- 3.83	- 7.4	- 12	0.2
Mn_2GS-Gln + ADP	$(- 8.47)_{calc}$	- 4.2	+ 14	0
Mn_2GS + ADP + Gln	$(-11.4)_{calc}$	-14.4	- 10	0.2
ADP-MgGS + Glu	- 3.81	- 7.7	- 13	0.2
ADP-P_i-MgGS + Glu	$\sim$ - 2.18	$\sim$ - 1.0	$\sim$ + 4	0.2
ADP-P_i-MgGS + Ala	$\sim$ - 2.53	+ 1.8	+ 14	0.2
Mn_2GS + AMP	$\sim$ - 5.0	-11.1	- 20	0.6
Mn_2GS + Trp	$\sim$ - 4.2	- 7.0	- 9	0

* Two equiv of Mn^{2+} must be bound per subunit for the expression of glutamine synthetase activity: one Mn^{2+} binds to a structural, activating site and one Mn^{2+} complexes to ADP at the catalytic site. For Mg^{2+}, the stoichiometry of binding is not well established.

There is synergism between the binding of substrates, L-glutamine and ADP, and the overall entropy of binding both substrates is negative whereas the entropies of binding ADP are positive and those for L-glutamine are negative. A list of these Mn-enzyme species in sequence from the most ordered to the most disordered state is: MnGS-Gln, ADP-MnGS-Gln, MnGS, and ADP-MnGS. Thus, the ternary complex is more ordered than the free, unliganded Mn-enzyme suggesting that a disruption in solvent or ligand structure (protein and/or water ligands) on binding ADP is overcome by the ordering effects of L-glutamine binding. The thermodynamic parameters and proton uptake for the binding of L-glutamate to the ADP-Mg-enzyme are the same as those for L-glutamine binding to the ADP-Mn-enzyme complex, suggesting similar binding mechanisms. Binding the feedback inhibitors AMP and L-tryptophan to the Mn-enzyme produces an ordering effect whereas the entropy change for binding the inhibitor L-alanine to the ADP-P_i-MgGS complex is positive. Finally, the calorimetric results on L-glutamate and L-alanine binding to the Mg-enzyme indicate that the biosynthetic reaction products (ADP + P_i) mimic the action of ATP on L-alanine binding but differ from that of ATP on L-glutamate binding. That is, there appears to be a synergistic effect between the binding of L-alanine and ADP + P_i and an antagonistic effect between L-glutamate and ADP + P_i.

(3) Studies of a reversible thermal transition in glutamine synthetase. (Investigator: A. Shrake). Measurement of an ultraviolet, temperature-difference spectrum of E. coli glutamine synthetase revealed the existence

of a reversible thermal transition in the temperature range of 20-65°. The shape of the difference spectrum and the magnitude of the effect are compatible with the perturbation of a single tryptophan residue per subunit. Melting curves were measured for adenylylated and unadenylylated enzyme with different levels of Mn^{2+} and Mg^{2+} present. The effect appears to be independent of the state of adenylylation and of the nature of the divalent cation present (Mn^{2+} or Mg^{2+}). However, the melting temperature (T_m) is proportional to the level of saturation of enzyme with divalent cation. For adenylylated and unadenylylated enzyme with both divalent cation sites/subunit saturated with Mn^{2+}, T_m is 50.5°. For unadenylylated enzyme with approximately one divalent cation site/subunit saturated with Mn^{2+} or Mg^{2+}, T_m is 46°. A T_m value for divalent cation-free enzyme will be measured also. Reversible thermal transitions are common in proteins containing a single polypeptide chain, but the finding of such a phenomenon with a dodecameric enzyme is noteworthy. Circular dichroism, light scattering, activity measurements and ultracentrifugation will be used to investigate the nature of the thermal transition in glutamine synthetase (e.g., local unfolding or dissociation of subunits). Using the Mn^{2+}-supported γ-glutamyl transfer reaction, Van't Hoff heats for the binding of substrates to the enzyme will also be measured and compared with heats measured directly in the calorimeter.

(4) Pyridoxylation of glutamine synthetase of E. coli. (Investigators: E. J. Whitley and A. Ginsburg). A specific labeling of phosphate binding sites of unadenylylated glutamine synthetase has been achieved by a reaction of the enzyme with pyridoxal 5'-phosphate at pH 8.1 (K_A^i = 700 M^{-1}), followed by borohydride reduction of the Schiff base (formed presumably with lysyl ε-amino groups). The loss in Mg^{2+}-dependent activity as a function of pyridoxamine phosphate (PMP) incorporation is linear; complete inactivation is produced by the incorporation of 3.0 equiv of PMP groups per subunit. However, the modified protein retains substantial Mn^{2+}-supported activity. The catalytic parameters summarized for native GS and the PMP-GS derivative (2.6 equiv of PMP groups/GS subunit) suggest that the catalytic site of each subunit is modified and that pyridoxylation markedly affects the affinity of the Mn-enzyme for nucleotide substrates.

Assay Method* & Metal Ion Support		V_{max} (U/mg) GS	V_{max} (U/mg) PMP-GS	Substrate Varied#	K_m (mM) GS	K_m (mM) PMP-GS
Biosynthetic	Mg^{2+}	40	7	ATP	0.25	0.78
				Glu	2.3	2.6
	Mn^{2+}	1	1	ATP	2×10^{-3}	4×10^{-2}
				Glu	0.04	0.32
Transfer	Mg^{2+}	33	5	ADP	0.05	0.15
				Gln	11.0	10.0
	Mn^{2+}	100	40	ADP	2×10^{-5}	1×10^{-3}
				Gln	3.0	4.0
				Arsenate	0.08	0.08

* Buffers are: 50 mM Tris-imidazole (pH 7.36) for the Mg^{2+}-supported biosynthetic assay at 50 mM $MgCl_2$; 50 mM Pipes (pH 6.5) for the Mn^{2+}-supported biosynthetic assay at 6 mM $MnCl_2$; 50 mM 3,3'dimethylglutarate (pH 7.57) for γ-glutamyl transfer assays with either 30 mM $MgCl_2$ or 0.4 mM $MnCl_2$.

For biosynthetic assays the substrates when not varied were fixed at 30 mM L-glutamate, 5 mM ATP, and 50 mM NH_4^+ in Mg^{2+} assays and at 100 mM L-glutamate, 5 mM ATP, and 100 mM NH_4^+ in Mn^{2+} assays. In transfer assays, the substrates when not varied were fixed at 150 mM L-glutamine, 0.4 mM ADP, 20 mM arsenate and 40 mM NH_2OH.

The pyridoxylated enzyme derivative is stable in the dark and to small amounts of irradiation. However, the PMP groups are photolyzed quickly by the intense light of the fluorometer during excitation at 330 nm.

The Mn-PMP-GS derivative has an absorption maximum at 326 nm; the protein bound PMP groups appear to be fully exposed to solvent since the absorption coefficient is the same as that reported for PMP-lysine. The covalently bound PMP groups have provided spectral probes for ligand interactions with the enzyme. Perturbations at 326 nm have been observed for interactions of the Mn-PMP-enzyme with ATP, ADP, AMP and P_i. Removal of Mn^{2+} ions from the PMP-enzyme with EDTA causes a large absorbancy change at 340 nm; in the presence of EDTA, the PMP absorption is perturbed by AMP or P_i but not by ADP or ATP. Only P_i perturbed the spectrum of the Mg-PMP-enzyme. Carbamyl phosphate, CTP, L-glutamate, or L-glutamine does not alter the spectrum of the Mn-PMP-enzyme although the spectrum of the ADP-Mn-PMP-enzyme-Glx complex is different from that of the ADP-Mn-PMP-enzyme complex. Monitoring the PMP absorption changes during ligand additions to the Mn-PMP-enzyme provides binding constants and information on interactions between binding sites. The spectral perturbations caused by ATP or P_i at pH 7.4 are opposite in direction to those caused by AMP or ADP. The combination of ADP + P_i does not produce the same perturbation as does ATP and additional

evidence has been obtained for AMP and ADP binding to separate sites on the Mn-PMP-enzyme. These studies are being continued to learn more about the topography of divalent cation and other ligand binding sites. Amino acid sequencing of PMP-peptides may be attempted later to obtain the primary structure at the subunit catalytic site.

(5) Kinetic studies on glutamine synthetase-metal ion interactions. (Investigators: J. B. Hunt and A. Ginsburg). Additional kinetic studies of protein-metal ion interactions are being performed. Unadenylylated glutamine synthetase and dye-metal ion complexes give information on the mechanism of Mn^{2+} interactions with the divalent cation-free enzyme. Studies of Me^{2+} binding reactions in the presence of pH-indicator dyes give kinetic data on the proton release from the protein. Analogous studies on the removal of Mn^{2+} from the enzyme by dipicolinic acid in the presence of a dye pH-indicator show the kinetics of proton uptake by the enzyme. These studies are in progress but already indicate the following: when glutamine synthetase is challenged with sufficient Mn^{2+} to saturate 2 subunit sites, Mn^{2+} is bound instantaneously and randomly to both sites (with about the same K_A^I). As the conformational change that is associated with Mn^{2+} binding to the tight n_1 site proceeds, the K_A^I increases to that value measured by equilibrium dialysis. Thus, the structural transition ($GS_{relaxed} \rightleftharpoons GS_{taut}$) is from a low- to a high-affinity form with Mn^{2+} (Mg^{2+} or Ca^{2+}) shifting the equilibrium to the high-affinity enzyme form. The slow proton release associated with Mn^{2+} binding to n_1 subunit sites results from a perturbation of an ionizable protein group which is not involved directly in binding Mn^{2+}.

(6) Studies on the polymerization of γG-immunoglobulin. (Investigator: A. Ginsburg). Immunoglobulin IgG has been purified from normal goat serum by DEAE-cellulose chromatography. Similarly purified IgG fractions from normal rabbit and human sera have been supplied by D. A. Zoph (LBP, NIAMDD). Various polymerization methods have been used, including heat and glutaraldehyde treatments and reactions with dimethylsuberimidate. Treated IgG samples are analyzed by electrophoresis on 5% polyacrylamide gels in the presence of 0.1% sodium dodecyl sulfate without and with β-mercaptoethanol also present to break interchain disulfide bonds. To date, the only treatment to produce significant amounts of polymerized IgG (an n-mer of 160,000 MW) was that with glutaraldehyde. In order to provide a means of specifically cross-linking the Fc region of IgG (leaving the antibody-antigen combining site free), the affinity of IgG fractions from different sources for Protein A will be tested. Once polymerization methods are worked out, affinity purified, carbohydrate-specific immunoglobulins (from the Section on Biochemistry, LBP, NIAMDD) will be used to obtain specific agglutinating activites.

Significance to Bio-Medical 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.

A general technique for producing a strongly agglutinating antibody from IgG would be valuable clinically as a replacement for types of rare sera or of sera that give only low positives in agglutination assays.

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, microcalorimetry, spectral, fluorescence, equilibrium binding, pH, electrophoretic, and kinetic techniques will be used.

(2) To study mutual interactions of divalent cations, substrates, and inhibitors with glutamine synthetase from E. coli. Pyridoxylation of the subunit catalytic site will be used to obtain information on the topography of divalent cation, substrate, and inhibitor sites.

(3) Thermal transitions of glutamine synthetase will be explored further, using activity assay, spectral, and differential scanning calorimetry techniques.

(4) In collaboration with the Section on Enzymes, LBP, NIAMDD, NIH, to produce an agglutinating activity in a goat IgG antibody directed against a human Le^b blood group hapten (Lacto-N-Difucohexaose I) by a polymerization reaction. Intermolecular cross-linking with glutaraldehyde, bifunctional imidoesters, and other reagents will be attempted.

Publications:

Caban, C.E., and Ginsburg, A.: Glutamine Synthetase Adenylyltransferase from Escherichia coli: Purification, Physical and Chemical Properties. Biochemistry, 15 , 1569-1580, 1976.

Shrake, A., Powers, D.M., and Ginsburg, A.: Calorimetric and Equilibrium Binding Studies of the Interaction of Substrates with Glutamine Synthetase of Escherichia coli. Biochemistry. 1977 (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 00205-22 LB

PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Role of Selenium in Anaerobic Electron Transport and Methane Biosynthesis

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

PI:	Thressa C. Stadtman	Chief, Section on Intermed. Metab. & Bioenergetics	LB	NHLBI
OTHER:	Joyce E. Cone	Staff Fellow (terminated 10/8/76)	LB	NHLBI
	Rafael Martin	Visiting Fellow (term. 9/10/76)	LB	NHLBI
	Belinda Seto	Staff Fellow (see indiv. report)	LB	NHLBI
	Hidehiko Tanaka	Staff Fellow (see indiv. report)	LB	NHLBI
	Vazhiyil Venugopalan	Visiting Fellow (since 5/5/77)	LB	NHLBI
	Jay B. Jones	Technical ass't & predoctoral student G.W. Univ. (see indiv. report)	LB	NHLBI
	Joe N. Davis	Research ass't (in charge of anaerobic laboratory, amino acid analyzers, gel electrophoresis, etc. - see indiv. report)	LB	NHLBI

COOPERATING UNITS (if any)

Blair Bowers, Ph.D., Laboratory of Cell Biology, NHLBI, NIH.

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Intermediary Metabolism and Bioenergetics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.5	3.5	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) The glycine reductase selenoprotein of C. sticklandii contains carbohydrate which may be located at the blocked amino terminus of the polypeptide chain. ^{75}Se-labeled protein synthesized by cells treated with antibiotics that inhibit glycosylation exhibits lower than normal biological activity suggesting the importance of glycosyl groups for interaction with the other proteins of the glycine reductase complex. The reconstituted glycine reductase complex (like the crude system) catalyzes the esterification of orthophosphate and forms ATP. The complete enzyme system is required for ATP synthesis. Antibodies to the selenoprotein partially inhibit its catalytic activity. Synthesis of the selenocysteine moiety of the selenoprotein from ^{75}Se-selenite is blocked by antibiotics that inhibit protein synthesis and also by rifampicin. These studies attempt to differentiate between a mechanism involving a post-translation modification of an existing residue in the polypeptide chain (e.g. serine) and direct introduction of the specific selenocysteine residue during chain elongation. A ^{75}Se-labeled amphoteric compound is produced by C. sticklandii during growth in media containing $Na_2{}^{75}SeO_3$. This compound serves as a source of selenium for synthesis of the selenoprotein and may be an intermediate.

PHS-6040

Project Description:

1. Anaerobic metabolism of certain amino acids with special reference 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 interreaction 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 additional components of the glycine reductase complex in order to determine the mechanism of the reaction and the coupled phosphorylation process.

2. Isolation and characterization of selenium-dependent and selenium-independent formate dehydrogenases of Methanococcus vannielii and Clostridium sticklandii.

   a. Determination of subunit structure, catalytic properties and metal ion stoichiometry of formate dehydrogenase that contain
      i. molybdenum and iron
      ii. molybdenum, iron and selenium
      iii. tungsten analogs of (i) and (ii).
   b. Characterization of pteridine-like "Factor 420"-dependent formate dehydrogenase-NADP reductase system of M. vannielii.
3. Mechanism of methane biosynthesis from acetate and formate and role of vitamin B_{12} and "Factor 420" in the process.

Major Findings:

(1) The glycine reductase complex of proteins that catalyzes the reductive deamination of glycine (eq. 1) is of metabolic importance as an electron sink for a number of amino acid fermenting clostridia.

$$\begin{matrix}CH_2COOH \\ | \\ NH_2\end{matrix} + R(SH)_2 + Pi + ADP \rightarrow CH_3COOH + NH_3 + R\begin{matrix}S \\ | \\ S\end{matrix} + ATP \qquad (1)$$

Detailed characterization of the protein components of this complex, one of which is a selenoprotein, have as their major aim to elucidate the mechanism of ATP synthesis in the reaction and the biochemical role of selenium in a oxido-reduction reaction of this type.

The amino acid composition of the glycine reductase selenoprotein (e.g. no tryptophan, high phenylalanine to tyrosine ratio and high content of acidic amino acids) explained the unusual electronic spectrum and acidity of the protein. Identification of the selenoprotein as a glycoprotein (by Dr. Joyce Cone) afforded an explanation of the differing molecular weight values obtained by molecular sieve chromatography and disc gel electrophoresis procedures. Also, the blocked amino terminus (established by Dr. Rafael

Martin) could be due to the presence of N-glycosyl groups linked to an N-terminal aspartate residue.

Studies on the biosynthesis of the selenoprotein showed that protein synthesis is essential for incorporation of labeled selenium and also for production of biologically and antigenically active material. Selenoprotein formation also is prevented by rifampicin, an antibiotic that blocks DNA-dependent RNA synthesis. Thus, the selenocysteine moiety of the selenoprotein may be introduced during de novo protein synthesis rather than as a post-translational modification. Should the Se moiety of other selenoproteins such as mammalian and avian glutathione proxidase also prove to be a selenocysteine residue then this may prove to be a more likely possibility.

In the presence of antibiotics that inhibit glycosylation and cell membrane formation (e.g. bacitracin and monensin) cells synthesize ^{75}Se-labeled selenoprotein with lower than normal biological and antigenic activity. This finding suggests that glycosyl groups are important for interaction of the selenoprotein with other proteins of the glycine reductase complex and also as antigenic determinants.

Studies in collaboration with Dr. H. Tanaka on the mechanism of ATP synthesis by glycine reductase show that the recombined highly purified protein components catalyze the esterification of one equivalent of ortho-phosphate and the incorporation of one ADP into ATP per mole of glycine reduced to acetate and ammonia. For these experiments homogenous seleno-protein and glycine reductase proteins B and C that were free of adenylate kinase were used. No liberation of ammonia or formation of ATP was observed except in the presence of all reaction mixture components. In preliminary experiments designed to detect a thio or selenophosphate derivative of the selenoprotein (a possible enzyme bound phosphate ester intermediate in the reaction) conditions have been worked out that allow reisolation of the biologically active form of the protein from $^{32}PO_4$ reaction mixtures under conditions that also should preserve an S-P ester intermediate.

(2-3) Work on the formate dehydrogenases of C. sticklandii and M. vannielii, investigated primarily by J. Jones, is described in his separate progress report. Both selenium-dependent and selenium-independent forms of formate dehydrogenase are present in M. vannielii.

Proposed Course of Research:

(1) Characterization of the active site of the glycine reductase selenoprotein particularly with regard to the location of the selenocysteine and cysteine residues. Since a tryptic peptide of about 1500-2000 daltons contains the selenocysteine residue, this can be prepared from protein alkylated with ^{14}C-iodoacetamide to see if the cysteine residues also are present in the same fragment. Selenium-77 labeled protein will be prepared for NMR studies if sufficiently sensitive instrumentation is available.

(2) In vitro systems which consist of C. sticklandii cell suspensions

treated with sucrose-lysozyme-EDTA or with detergents such as Triton X-100 catalyze the uptake of ^{75}Se-labeled selenite and the formation of biologically active ^{75}Se-labeled selenoprotein. Such systems will be studied to determine the mode of biosynthesis of the selenocysteine moiety of the glycine reductase selenoprotein and also of formate dehydrogenase.

(3) Collaborative experiments with Dr. Belinda Seto on antigenic determinants of the selenoprotein and nature of the glycosyl groups and the mechanism of their attachment to the protein.

(4) Reconstitution of the glycine reductase system from purified protein components; identification of the enzyme-bound precursor of acetate and the phosphorylated intermediate that is converted to ATP.

(5) Nature of early steps in the reduction of carbon dioxide and formate to methane and role of "pteridine-like" "factor-420" in the coupling of formate oxidation to methane biosynthesis. Also studies on the identity of "factor 420".

Publications:

Jay B. Jones and T. C. Stadtman. Methanococcus vannielii: Growth and Metabolism of Formate. In "Microbial Production and Utilization of Gases" E. Goltze KG, Göttingen, p. 199-205 (1976)

T. C. Stadtman, J. E. Cone, R. Martin del Rio, J.B. Jones and B. Seto. Selenoenzymes of Bacteria. Proc. of Symp. on Selenium-Tellurium in the Environment. Univ. of Notre Dame, Notre Dame, Ind. p. 226-233 (1977)

J.E. Cone, R. Martin del Rio, J.N. Davis and T.C. Stadtman. Chemical Characterization of the Selenoprotein Component of Clostridial Glycine Reductase: Identification of Selenocysteine as the Organoselenium Moiety. Proc. Nat. Acad. Sci. USA, 73, 2659-2663 (1976).

Belinda Seto and T.C. Stadtman. Purification and Properties of Proline Reductase from Clostridium sticklandii. J. Biol. Chem. 251, 2435-2439 (1976)

J.B. Jones and T.C. Stadtman. Methanococcus vannielii: Culture and Effects of Selenium and Tungsten on Growth. J. Bacteriol. 130, 1404-1406 (1977).

J.B. Jones, Blair Bowers & T.C. Stadtman. Methanococcus vannielii: Ultrastructure and Sensitivity to Detergents and Antibiotics. J. Bacteriol. 130, 1357-1363 (1977).

T.C. Stadtman. Selenium-dependent Clostridial Glycine Reductase. Methods in Enzymology. (in press).

J.E. Cone, R. Martin del Rio and T.C. Stadtman. Clostridial Glycine Reductase Complex: Purification and Characterization of the Selenoprotein Component. J. Biol. Chem. (in press).

T.C. Stadtman. Biological Function of Selenium. Nutrition Reviews (in press for August or September, 1977 issue).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00206-18 LB

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Stereochemical Studies of Enzymatic Reactions

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

PI:	Lin Tsai	Chemist	LB	NHLBI
OTHER:	Elizabeth Caveney	Chemist (terminated 6/3/77)	LB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Section on Intermediary Metabolism & Bioenergetics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.7	1	0.7

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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) The rearrangement catalyzed by coenzyme-B_{12} dependent enzyme, αMG mutase, and an isomerase was studied with 2'-^{14}C,2'-^{3}H-α-methylene-glutaric acid. A partially purified enzyme preparation was found to convert α-methyleneglutarate to dimethylmaleate with no significant loss of tritium.

(2) A stereospecific synthesis of 2(R,S),5(S,R)-diaminohexanoic acid was achieved. This isomer was found to be the inactive isomer for the enzyme D-α-lysine mutase. Therefore, the enzymically active isomer must be 2R,5R-diaminohexanoic acid.

PHS-6040
(Rev. 10-76)

Project Description:

Objective 1: For the study of the steric course of the rearrangement catalyzed by αMG mutase, it is important to establish conditions that minimum hydrogen exchange occurs since any exchange of hydrogen would mean loss of stereospecificity.

Major Findings:

In order to test the extent of hydrogen exchange during the αMG mutase-MIT isomerase reactions, 2'-^{14}C,2'-^{3}H-α-methyleneglutaric acid was prepared with different $^{3}H/^{14}C$ ratios to be used as substrate. After many attempts, a fraction from a Sephadex G-150 column was found to have the appropriate proportion of mutase and isomerase activities that gave rise to 4-10% conversion of α-methyleneglutarate to dimethylmaleate with no significant loss of tritium. Some typical results were as follows:

	$^{3}H/^{14}C$ Ratios	
Substrate αMG	Product DMM	Unreacted αMG
3.482 ± .080	3.553 ± .024	3.466 ± .018
3.478 ± .060	3.367 ± .050	3.537 ± .021
	3.528 ± .030	3.542 ± .017
6.730 ± .091	6.843 ± .087	6.660 ± .079
6.710 ± .097	6.720 ± .092	6.719 ± .105
6.784 ± .092	6.883 ± .154	-
	6.986 ± .084	-

Although all of these values are within statistical errors indicating none or very little hydrogen exchange, the slightly higher $^{3}H/^{14}C$ ratios in the product DMM than the substrate αMG in most of the experiments is rather puzzling. This might be due to the slightly different degree of purity of the substrate and the product samples or to some inadequacy of the method employed for the radioactivity determination.

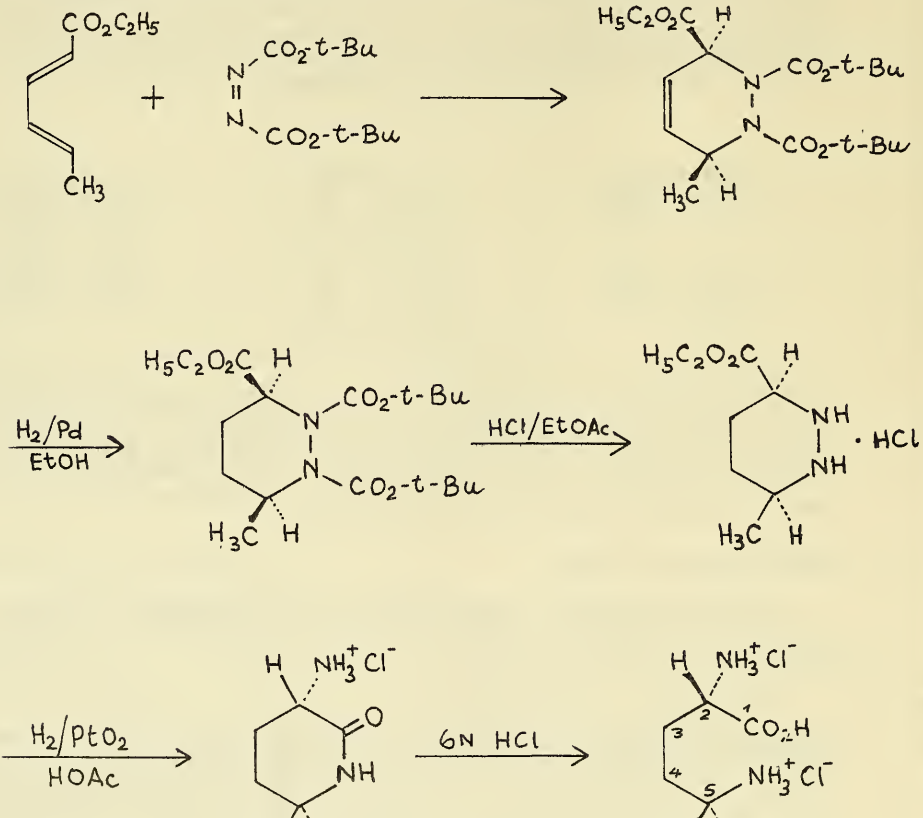
Proposed course of action:

To examine in detail various factors of the experimental conditions so as to establish a definitive procedure for future experiments using a stereospecifically ^{3}H labeled substrate.

Objective 2: The rearrangement of D-α-lysine to 2,5-diaminohexanoic acid is catalyzed by a coenzyme-B_{12} dependent enzyme, D-α-lysine mutase. 2,5-Diaminohexanoic acid possesses two asymmetric centers, hence there are two diastereoisomeric pairs having 2(R,S),5(S,R) and 2(R,S),5(R,S) relationship for the two amino groups. To study the stereochemistry of the D-α-lysine mutase reaction, it is desirable to establish the relative configuration of the two amino groups in 2,5-diaminohexanoic acid produced by the enzyme.

Major Findings:

2,5-diaminohexanoic acid was prepared by a straightforward synthesis which yielded a mixture of diastereoisomers. Various attempts to separate the isomers were met with only minor success. The benzamide derivatives of the mixture could be separated with difficulty into two components by preparative TLC. These were characterized by the spectral properties as the two diastereoisomeric forms of 2,5-diaminohexanoic acid. However, it was not possible to assign unambiguously the relative configuration of the two isomers on the basis of their ^{1}H-NMR spectra. To resolve this problem, a stereospecific synthesis was designed and accomplished as outlined in SCHEME (stereochemical representation of one enantiomer only).



SCHEME

This synthesis took advantage of the well-recognized cis-addition in the Diels-Alder reaction in the first step, thus the relative configuration of the two nitrogen substituents was established at the outset. All the subsequent steps did not involve any change of either asymmetric center. Therefore, the final product, 2,5-diaminohexanoic acid, must have the 2(R,S),5(S,R)-relationship for the two amino groups.

We had established previously (Dr. T.C. Stadtman's expt.) that the enzymically active isomer of 2,5-diaminohexanoic acid was derived from the high-melting benzamide derivative. In order to make the correlation, the 2(R,S),5(S,R)-isomer was converted to the benzamide derivative, whose ^{1}H-NMR spectrum and m.p. were identical with those of the enzymically inactive isomer. Thus the enzymically active isomer must have the 2(R,S),5(R,S)-relationship, and consequently, the absolute configuration of enzymic product is 2R,5R-diaminohexanoic acid since during the mutase reaction, the asymmetric center at C_2 of the substrate, D-lysine (2R-lysine), is not involved.

Proposed course of action:

To synthesize the enzymically active isomer of 2,5-diaminohexanoic acid and to verify its activity for the enzyme.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00208-04 LB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Electron transport processes associated with proline reduction in Clostridium sticklandii.

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

PI:	Belinda Seto	Staff Fellow	LB NHLBI
OTHER:	Thressa C. Stadtman	Chief, Section on Intermediary Metabolism & Bioenergetics	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 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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 major findings suggest that there is a copper protein which can transfer electrons from sodium dithionite to proline reductase during the reduction of D-proline. Additional electron transport protein(s) are involved in coupling NADH to the terminal portion of this reduction system.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: (1) to isolate and characterize the electron transport components associated with proline reduction (2) to reconstitute the complete proline reduction system with purified components.

Major Findings:

(1) A copper containing protein which can be reduced by sodium dithionite and then in turn reduces proline reductase has been purified to homogeneity. This protein has a molecular weight of 86,000 and consists of two identical subunits of 43,000 daltons.

(2) Presently, it is possible to reconstitute a proline reduction system. The system consists of the following constituents: sodium dithionate as electron donor, Cu protein, proline reductase and D-proline. NADH, the physiological electron donor, fails to couple in the above system. The evidence suggests additional electron transport components are involved between NADH and the terminal portions of this electron transport system.

(3) In addition, I am involved in a cooperative project with Dr. T. C. Stadtman. We are interested in characterizing the antigenic determinants of the selenoprotein of glycine reductase in Clostridium sticklandii. This is an alternative approach for determining the catalytic significance of Se in this protein. Antiserum against the selenoprotein was prepared and immobilized on AH-sepharose 4B. This immunoadsorbant chromatography system will be used to study antigenic properties of various peptides which resulted from tryptic digestion of the selenoprotein. In addition, peptides in which the Se moiety has been alkylated are also under investigation.

Publications:

T. C. Stadtman, J.E. Cone, R. Martin del Rio, J. B. Jones and B. Seto. Selenoenzymes of Bacteria. Proc. of Symp. on Selenium-Tellurium in the Environment. Univ. of Notre Dame, Notre Dame, Ind. p. 226-233 (1977)

Belinda Seto and T. C. Stadtman. Purification and Properties of Proline Reductase from Clostridium sticklandii. J. Biol. Chem. 251, 2435-2439, (1976).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00211-04 LB

PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)
Regulation of Glutamine Synthetase

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

PI:	E. R. Stadtman	Chief, Laboratory of Biochemistry	LB NHLBI
OTHER:	P. Z. Smyrniotis	Chemist	LB NHLBI
	Joe N. Davis	Biochemist	LB NHLBI
	Mary Wittenberger	Laboratory Technician	LB NHLBI

COOPERATING UNITS (if any)

None.

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.25	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)

The binding of up to one equivalent of CIBA chrome F-3GA Dextran to each subunit of E. coli glutamine synthetase (GS) results in a marked blue shift in the dye spectrum. The nucleotides, ATP, CTP, ADP, and AMP compete with the dye for binding GS suggesting that the dye binds to the nucleotide catalytic site and possibly also to the AMP allosteric site. The spectrum of the change elicited by dye binding varies with ionic strength, pH, and the concentrations of different allosteric effectors. From the ligand concentration dependency of the allosteric spectral purturbation it appears that ligand binding induces a conformational change in the enzyme. Marked differences in the spectral changes caused by dye binding to relaxed (metal-free), taut (metal bound), and to dissociated subunits of GS are the basis of a convenient method to study the effects of substrates and allosteric ligands on the kinetics of the relaxation and subunit dissociation of GS. By means of affinity chromotography on CIBA chrome G-3GA sepharose columns, it has (for the first time) been possible to separate hybrid molecular forms of GS containing different amounts of adenylylated and unadenylylated subunits.

5K

Project Description:

It has been established by other workers that the binding of CIBA chrome dextran F-3GA to the nucleotide fold of several dehydrogenases and kinases causes a marked shift in the dye spectrum in the region of 700 to 400 nm. Moreover, the ability of nucleotides to compete with dye binding has been used to estimate the binding constants of various nucleotides to these enzymes. Since preliminary studies showed that the dextran dye also binds to E. coli glutamine synthetase, the present project was initiated to determine if the spectral changes associated with dye binding could be utilized to measure interactions of substrate and allosteric effectors to glutamine synthetase, and to measure changes in protein conformations associated with interconversion of the enzyme between relaxed and taut forms. In addition it was hoped that chromatography of glutamine synthetase on dye-affinity columns could be used to separate enzyme forms containing different numbers of adenylylated subunits.

Major Findings:

(1) Use of CIBA chrome blue to measure glutamine synthetase (GS) - ligand interactions. Binding of CIBA chrome F-3GA to glutamine synthetase results in a marked change in its absorption spectrum. The spectral changes are reversed by the addition of various nucleotides including ATP, CTP, ADP and AMP suggesting that the dye binds to the nucleotide substrate site. From spectral titration studies and also from equilibrium binding studies involving ultracentrifugation of the enzyme and the dye-enzyme complexes, it was established that only one equivalent of dye is bound per subunit of enzyme. From equilibrium binding measurements and from the kinetics of the dye inhibition of enzyme activity it was established that the stability constant of the dye-enzyme complex is about 0.9 μM. The overall amplitude of the spectral change elicited by the addition of a 2-fold excess of dye to the enzyme all at once is 1.4 times greater than that obtained by stepwise titration of the enzymes with dye to the same final dye concentrations. This and the fact that there is a gradual shift in the λ max of the difference spectrum elicited by progressive titration of the enzyme with dye, but not when excess dye is titrated with enzyme, indicates that binding of dye to only a few subunits in a enzyme molecule induces a conformational change that affects the environment of the binding site. It was noted that qualitatively, different spectral changes are obtained when dye binds to enzyme in the presence of and absence of various salts and allosteric effectors, but it remains to be determined whether these differences are due to differences in protein conformations at the dye binding sites or reflect changes in the spectrum of the dye itself.

(2) Use of CIBA chrome F-3GA to probe changes in conformation associated with relaxation and dissociation of the enzyme. There are qualitative and quantitative differences in the spectral changes obtained when dye binds to the relaxed (divalent cation-free) enzyme, the taut enzyme (containing Mn^{2+} or Mg^{2+}) and the dissociated subunits. Preliminary studies show that these spectral differences can be utilized to explore the effects of pH, ionic strength, urea, detergents and the concentrations of allosteric effectors and substrates on the relaxation and subunit dissociation of the enzyme.

(3) Separation of partially adenylylated enzyme molecules. By taking advantage of the fact that higher concentrations of ADP are required to displace dye from adenylylated enzyme than from unadenylylated enzymes, it has been possible with affinity chromatography on CIBA chrome-F-3GA-sepharose columns to separate unadenylylated and fully adenylylated enzyme from mixtures of the two. Preliminary experiments indicate that with refinements this technique can be used to separate molecular species of enzyme that differ by only 1 or 2 in the number of adenylylated subunits they contain. Such separations will permit further studies on the effects of heteroeogous subunit interactions in hybrid molecules on various catalytic and physical properties of the enzyme.

Significance to Biomedical Research:

Whereas defects in the genetic machinery of the cell may be primarily responsible for most metabolic diseases, including those resulting in abnormal cell growth (cancer), it is evident that the manifestiation of these genetic defects is a consenquence of specific impairments of important regulatory functions. The possibilities of developing therapeutic procedures to selectively correct genetic defects at the gene level are remote. However, with a better understanding of the mechanisms of cellular regulation, logical approaches to the developing effective therapeutic procedures at the level of cellular regulation will become available. To this end, The present study and other related studies in this laboratory are designed to gain a better understanding of basic principles that are utilized in the regulation of enzyme activity.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 00212-06 LB

PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Biochemical Genetics of NH_3-Assimilatory Enzymes in E. coli K_{12}

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

PI: Mary Anne Berberich Chemist LB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SE
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	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)

(1) Selection of, and genetic studies on, mutants with regulatory effects on glutamine synthetase and/or glutamate synthase and/or glutamate dehydrogenase in E. coli K_{12}.

(2) Studies on the mechanism of resistance to various substrate analogues and inhibitors of these several enzymes.

(3) Studies on the manifestation of regulatory interrelationships among these NH_3-assimilatory enzymes.

Project Description:

Objectives: (1) To develop methods to study possible regulatory interrelationships among the NH_3-assimilatory enzymes in E. coli K_{12}. (2) To elucidate the mechanism of derepression or induction of glutamine synthetase in E. coli K_{12}. (3) To isolate, and characterize biochemically, mutants in which the regulation of glutamine synthetase, glutamate synthase, and glutamate dehydrogenase are affected individually or collectively and to conduct genetic experiments for the purpose of locating these mutations on the E. coli chromosomal map.

Major Findings:

It has been shown that glutamine synthetase is not directly involved in the repression of glutamate dehydrogenase. Nor is it directly involved in the repression of glutamate synthase.

Physiological studies with wild-type E. coli and E. coli K_{12} have established growth conditions where regulatory effects are amplified for the group of NH_3-assimilatory enzymes. A N/C ratio of 10/1 is necessary to observe the repressed state of glutamine synthase and as the N/C ratio approaches 1, glutamine synthase approaches half-maximal levels. A N/C ratio of 1.21 is a good ratio to observe glutamine synthetase under conditions of rapid log-phase growth.

An extensive list of putative inducers was examined to test the idea that glutamate, in the absence of nitrogen, would become a "dead end" compound for glutamine synthetase and, by remaining bound to the enzyme, generate a conformation optimal for autogenous regulation. Alternatively, in the absence of nitrogen, the glutamate could be converted to a compound recognizable as a signal that the nitrogen supply was low. Of the compounds tested, only 2-methyl glutamate consistely raised the levels of glutamine synthetase, glutamate synthase, and glutamate dehydrogenase in a co-ordinate fashion. Cyclic-AMP enhances the positive effects of 2-methyl glutamate. Particularly noticable is the absence of a decrease in the level of glutamate synthase at cyclic-AMP concentrations of 5 mM. Similar results were obtained using permeablized cells.

Because chloroketone (5-chloro-4-oxo-L-norvaline, CONV) is an inhibitor of aminotransferases but not of glutamine synthetase or of glutamine synthetase adenylyltransferase system, the compound was examined with growing cells in an attempt to find a regulatory mutant. Glutamate synthetase is almost completely inhibited in vivro by 0.2 mM chloroketone, whereas glutamine synthetase is unaffected and glutamate dehydrogenase is affected to approximately 75%. Chloroketone-resistant mutants have been obtained from both wild-type and a GDH^- mutant. After growth in a glucose/NH_4Cl medium, assay of some of the isolates from wild-type shows glutamine synthetase, glutamate synthase, and glutamate dehydrogenase decreased by about half. Another isolate from wild-type shows a low level of glutamine synthetase but an apparently normal level of glutamate synthetase. Isolates from the GDH^- strains show increased levels

of glutamate synthase (ca. 3-4 times) and glutamine synthetase (1.5 to 2 times) when compared with the GDH^- controls. These isolates had not reverted to GDH^+.

Proposed Course of the Project:

(1) The chloroketone-resistant mutants which display effects on the NH_3-assimilatory enzymes will be genetically mapped.

(2) Studies on the cyclic-AMP enhancement of the 2-methyl glutamate effect will be pursued.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00214-03 LB

PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)
Iron and Coenzyme Dependent Inactivation of Klebsiella Glutamine Synthetase

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

PI:	Richard Fulks	Staff Fellow	LB NHLBI
Other:	Earl R. Stadtman	Chief, Lab of Biochemistry	LB NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	1.1	0.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

In the presence of iron and DPN or TPN extracts of Klebsiella aerogenes convert glutamine synthetase into an enzymatically inactive but immunologically reactive species. The inactivation of glutamine synthetase in extracts is inhibited by antimycin A, anaerobiosis, and sulfhydryl reactive compounds, but not by the serine protease inhibitors PMSF, TSF, or pentamidine isethionate.

A nondialyzable extract component is also required for glutamine synthetase inactivation. The nondialyzable inactivating activity is heat sensitive and precipitates with $(NH_4)_2SO_4$ (80% saturated, 0°), but does not precipitate with streptomycin sulfate (3 g/100ml). Following ultra-filtration, glutamine synthetase inactivating activity is found in extract fractions of MW exceeding 50,000.

PHS-6040
(Rev. 10-76)

Project Description:

Resting suspensions of K. aerogenes inactivate glutamine synthetase (GS) by a mechanism that is energy-dependent (stimulated by glucose and inhibited by dinitrophenol) and pH-dependent but independent of protein synthesis (see previous annual reports). This inactivation is accompanied by loss of glutamine synthetase immunoreactive material, a result consistent with (although not proof of) degradation of the enzyme. Cell-free extracts inactive glutamine synthetase under conditions in which purified glutamine synthetase is stable. This report is concerned with characterization of the cell-free glutamine synthetase inactivation.

Major Findings:

1. Metals and Metal Chelators

The metal chelators EDTA (added as neutralized EDTA or Mg-titriplex) and o-phenanthroline (0.1 or 1 mM) inhibit GS inactivation in extracts by about 90%, suggesting that inactivation is metal-dependent.

When added at concentrations equal to the chelator concentrations, Ni++ completely reverses the inhibition caused by either EDTA (1.3 mM) or o-phenanthroline (0.1 mM). However, in the absence of chelator, Ni++ has no effect on GS inactivation when added at 10^{-6}, 10^{-5}, or 10^{-4} M to undialyzed extracts, or when added at 10^{-4} M to dialyzed extracts. At 10^{-3} M, Ni++ inhibits GS inactivation by 50-70%.

Co++ is about 80% effective in reversing the inhibition of GS inactivation caused by EDTA, and about 60% effective in reversing o-phenanthroline inhibition. As is true for Ni++, Co++ has no effect on GS inactivation when added to extracts at 10^{-6}, 10^{-5}, or 10^{-4} M. At 10^{-3} M, Co++ inhibits GS inactivation by 80-90%.

Fe++ or Fe+++ (.011 or 0.17 mM) reverses the inhibition of GS inactivation caused by o-phenanthroline (0.11 mM), but 1.3 mM Fe++ does not reverse the inhibition caused by 1.3 mM EDTA and 1.5 or 3.0 mM Fe+++ does not reverse the inhibition caused by 1.0 mM EDTA. Nor is EDTA (1.1 mM) inhibition reversed when Zn++, Cd++, Co++, or $MoO_4^=$ (1.1 or 1.7 mM) is added along with the Fe+++ (1.7 mM). A satisfactory explanation has not yet been found for the fact that iron reverses the inhibition of o-phenanthroline but not the inhibition of EDTA.

Reversal of o-phenanthroline inhibition by Fe++ or Fe+++ (0.1 mM) is accompanied by an additional stimulation of GS inactivation. Iron stimulates GS inactivation in extracts in the absence or presence of o-phenanthroline, in the absence or presence of Mg++ (2 mM), and in dialyzed or undialyzed extracts, but iron does not stimulate the inactivation of partially purified GS (67 units/mg) incubated alone or with boiled extract. Higher concentrations of Fe+++ (0.5, 1.1, or 5.5 mM) also stimulate GS inactivation in extracts, but 0.1 mM is just as effective.

When added to extracts in the absence of other metal, Cu++ (1.3 mM) or Zn++ (0.1, 1, or 1.3 mM) also stimulates GS inactivation. However, when added to extracts containing 2 mM Mg++, Cu++ (0.1 mM) inhibits GS inactivation by 85%, whereas Zn++ has no effect at 0.1 mM but at 5.5 mM inhibits GS inactivation by 100%. When added at concentrations equal to or 50% greater than the chelator concentration, neither Cu++ nor Zn++ reverses the inhibition of GS inactivation caused by o-phenanthroline (0.1 mM) or EDTA (1 mM). 3 mM Cu++ does reverse EDTA (1 mM) inhibition, and has an additional stimulatory effect on GS inactivation, but 3 mM Cu++ rapidly (in less than one hour) and completely inactivates partially purified GS, either in the presence or absence of 1 mM EDTA.

In extracts incubated in the absence of chelator, Mn++ (1 mM), Cd++ (1 mM), or Mg++ (24 mM) inhibits GS inactivation by 90-100%, and 1 mM Ca++ causes about 40% inhibition. None of these four metals reverses the inhibition of GS inactivation caused by chelators.

Conclusion: Considered together, these studies with metals and metal chelators suggest that for enzymatic inactivation of glutamine synthetase in Klebsiella extracts, iron is specifically required.

2. Nucleotides and Related Compounds

Experiments in cell suspensions (reported previously) suggest that GS inactivation requires energy. It is therefore of interest to examine the effect of ATP of GS inactivation in extracts. When extracts are dialyzed for 16-19 hours and then incubated in the presence of 0.1 mM Fe+++, ATP (1.1 mM) stimulates GS inactivation. ATP has little effect when added to extracts in the absence of iron, or when added to partially purified GS, either in the presence or absence of iron. The K_m for ATP for stimulation of GS inactivation in dialyzed extracts is approximately 0.1 mM.

CTP is just as effective as ATP in stimulating GS inactivation in the dialyzed extracts, indicating that nucleotide stimulation is not specific for ATP. ADP can also substitute for ATP, but it is possible that ADP stimulation results from ATP production due to adenylate kinase activity of the extract. However, such an explanation cannot account for the stimulation of GS inactivation caused by pyrophosphate, 3'AMP, 5'AMP, and the ATP analogue adenylyl imidodiphosphate, all of which are 50-80% as effective stimuli as ATP. Phosphate and 5'adenosine monosulfate are 20-30% as effective as ATP in stimulating GS inactivation, whereas adenosine and adenine have no effect. All compounds were tested at 1.1 mM in the presence of 0.1 mM Fe+++ and 1.8 mM Mg++.

3. Coenzymes

Although Fe+++ and a nucleotide stimulate GS inactivation in extracts dialyzed for 16-19 hours, in extracts dialyzed for 2-3 days GS inactivation depends upon the addition of a coenzyme such as DPN. DPN-stimulated GS inactivation does not require a nucleotide but does require iron and a heat sensitive nondialyzable extract component, in addition to the substrate GS. DPN-stimulated GS inactivation has a K_m for DPN of 0.1 mM.

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TPN is just as effective as DPN in stimulating GS inactivation in extensively dialyzed extracts, regardless of whether the coenzymes are added as the oxidized forms (1.1 mM) or as the reduced forms (0.1 or 1.1 mM). At 1.1 mM, the reduced forms results in about 40% greater stimulation of GS inactivation than the oxidized forms.

The DPN analogue 3-acetylpyridine DPN stimulates GS inactivation as effectively as DPN, but the analogue 3-pyridinealdehyde DPN is about 40% less effective than DPN. Both analogues differ from DPN at a region close to the site involved in DPN-dependent oxidations and reductions.

CoA is about 50% as effective a stimulus as DPN, whereas FMN and FAD have no effect.

All coenzymes were tested in extracts dialyzed for at least 40 hours and then incubated in the presence of Fe+++ (0.1 mM) and absence of nucleotide. The one exception to this was TPNH, which was tested in the presence of 0. 15 mM Fe+++ and 1.1 mM ATP. ATP (1.1 mM) has no effect when added to dialyzed extracts incubated in the presence of 0.1 mM Fe+++ and 1.1 mM DPN.

4. Inhibitors

a. Protease Inhibitors

GS inactivation in extracts is not accompanied by loss of GS immunoreactive material, as determined by either gel immunodiffusion or quantitative immunoprecipitation. Immunoreactivity is retained even when extracts are incubated in the presence of Fe+++ (0.1 mM), ATP (1.1 mM), and DPNH (1.1 mM). Retention of immunoreactivity excludes the possibility that GS is completely degraded to amino acids, but does not eliminate the possibility of limited proteolysis. To test for protease involvement, the effects of protease inhibitors on GS inactivation in extracts was determined.

PMSF (1.1 or 5.5 mM), TSF (1.1 mM) or pentamidine isethionate (1.1 mM), each of which inhibits certain serine proteases, have no effect on GS inactivation in extracts.

TPCK (1.1 mM) or TLCK (1.1 or 5.5 mM) inhibits GS inactivation by 30-50%. These compounds inhibit some serine proteases, but they inhibit other enzymes, such as papain, by interacting with essential sulfhydryl groups.

b. Sulfhydryl-Reactive Compounds

N-ethylmaleimide (0.5 mM), iodoacetamide (1.1 mM), iodosobenzoic acid (0.5 mM), and p-chloromercuriphenyl sulfonic acid (1.1 mM) each inhibits GS inactivation in extracts by about 90%. Inhibition caused by iodosobenzoic acid or p-chloromercuriphenylsulfonic acid is reversed by equimolar concentrations of dithiothreitol.

c. Antimycin A and Anaerobiosis

Antimycin A (1.3 mM), which interferes with cytochrome electron transport, inhibits GS inactivation by about 90%. Perfusion of extracts with argon or helium to remove oxygen also inhibits GS inactivation. An oxygen requirement could explain the fact that during aerobic incubation of extracts GS inactivation is stimulated by shaking.

Interpretation: The preceeding inhibitor studies suggest that the inactivation of GS in extracts involves sulfhydryl groups and cytochrome-mediated oxidation, but not serine proteases.

5. When an extract that has been incubated to inactivate its GS is mixed with an equal portion of unincubated extract, the GS activity of the mixture is equal to the average of the activities of the unmixed extracts. This result argues against the possibility that inactivation of GS in extracts results from the production of a GS inhibitor.

6. Extract Fractionation

In addition to Fe+++, a coenzyme, and the substrate GS, GS inactivation in extracts requires a heat-sensitive nondialyzable extract component. This nondialyzable inactivating activity remains in the supernatant extract fraction after the addition of streptomycin sulfate (3g/100ml) and centrifugation to precipitate nucleic acids, but is found in the precipitate when the majority of extract protein is precipitated with $(NH_4)_2SO_4$ (80% sat., 0°).

Fractionation of the extract with $(NH_4)_2SO_4$ requires high centrifugal force (e.g., 78,000 x g) to achieve good separation of precipitate and supernatant fractions. Good separation is not achieved by centrifugation at 27,000 x g for one hour, with or without pretreatment of the extract with streptomycin, with or without the addition of Triton to reduce extract viscosity, or when different buffers are used. Thus $(NH_4)_2SO_4$ fractionation may not provide a convenient initial step for purification of the inactivating activity.

7. Molecular Weight

GS inactivating activity is found in the high molecular weight fraction, and not in the filtrate, when extracts are fractionated with filters that retain species of MW exceeding 30,000 or 50,000. When 300,000 MW filters are used, the filtrate contains some GS inactivating activity.

Publications: None.

GK

Proposed Course of Action

(1) Extracts incubated in the presence of iron and a coenzyme (oxidized or reduced DPN or TPN) convert GS into an enzymatically inactive but immunologically reactive species, which can be precipitated using the zinc procedure for GS purification. Isolation and characterization of this enzymatically inactive species may provide insight regarding the mechanism of inactivation.

(2) Isolate and characterize the heat-sensitive, nondialyzable, iron and coenzyme dependent GS inactivating activity.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 00215-02 LB

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Regulation of Membrane-bound Phosphofructokinase in Tetrahymena pyriformis

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

PI:	Paulette W. Royt	NIH Postdoctorate Fellow	LB	NHLBI
Other:	Earl R. Stadtman	Chief, Lab. of Biochem.	LB	NHLBI

COOPERATING UNITS (if any)

NIAID, NIH

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Section on Enzymes

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NHLBI, NIH, Bethesda, Maryland 20014

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) In Tetrahymena pyriformis, phosphofructokinase (PFK) is membrane-bound. Enzyme activity is solubilized by treatment of membranes with Triton X-100 or by high ionic strength in the presence of a chelator. The solubilized enzyme has an approximate molecular weight of 300,000 daltons. Membrane-bound PFK is inactivated when incubated with MgATP, NaF, and the 100,000 x g supernatant of a crude extract. The supernatant factor needed for inactivation is heat labile, non dialysable, acid precipitable and $(NH_4)_2SO_4$ precipitable. It has been partially purified by protamine sulfate precipitation, $(NH_4)_2SO_4$ precipitation, DE-52 column chromatography and gel filtration. The rate of inactivation is dependent on the concentration of the supernatant factor and that of MgATP. Adenosine, phosphate, pyrophosphate, cyclic AMP, or AMP did not replace the requirement for ATP, whereas ADP did. Experiments using radioactive ATP revealed that ATP binds very tightly to a membrane protein that is smaller than undissociated PFK. The inactive enzyme cannot be reactivated by dialysis, gel filtration, or treatment with acid phosphatase, alkaline phosphatase, or snake venom phosphodiesterase. Three protease inhibitors fail to affect the loss of activity. The irreversible ATP-dependent inactivation of PFK may be due to a ligand-induced conformational change of the enzyme, or possibly to a covalent modification of the enzyme.

Project Description:

In bacterial cells, plant cells, and higher animal systems, phosphofructokinase (PFK) is a soluble enzyme. In the ciliated protozoan, Tetrahymena pyriformis, PFK is instead membrane-bound. This project describes (1) the solubilization and characterization of membrane-bound PFK from Tetrahymena and (2) an in vitro ATP-dependent inactivation of membrane-bound activity.

Major Findings:

1. Solubilization and Characterization of PFK from Tetrahymena. Following sonication of cells for 60 sec and centrifugation of the extract for 1 hr at 100,000 x g PFK activity is found almost exclusively in the pellet fraction. If any enzyme activity is detected in the supernatant fraction, its level is insignificant. Also, after gently homogenizing cells and fractionating the extract on discontinuous sucrose gradients, all PFK activity is localized in the particulate fractions; no activity is found in the microsomal supernatant. By this technique, activities of four other glycolytic enzymes, pyruvate kinase, aldolase, hexokinase and phosphoglucose isomerase are found primarily in the microsomal supernatant indicating the soluble nature of these enzymes, and suggesting that PFK is not nonspecifically trapped in membrane vesicles. The distribution of PFK on the sucrose gradients coincides with that of lactic dehydrogenase and phosphoenolpyruvate carboxylase, two enzymes presumably bound to mitochondria in Tetrahymena. Activity of fructose diphosphatase could not be found on the sucrose gradients or in extracts of sonicated cells.

Membrane-bound PFK can be solubilized by incubating pellets in buffer containing Triton X-100. An approximate ratio of 10 μmoles of Triton to 1 mg membrane protein solubilizes 95% of the original PFK activity. Approximately 200% of the original activity is recovered in nonsedimentable form, indicating the release of latent enzyme or an activation of activity.

Incubation of membrane pellets in high ionic strength (NaCl or KCl) in the presence of EDTA also results in the solubilization of PFK activity as well as an activation of activity. However, under these conditions, release of the enzyme from the membrane is limited. Omission of chelator from the buffer resulted in a greater release of enzyme from the pellet but a lesser recovery of activity in the supernatant.

Chelators alone or increasing pH failed to solubilize membrane-bound activity.

Triton solubilized PFK does not inactivate upon removal of detergent by Bio Beads SM-2 or by gel filtration, nor does the detergent-free enzyme aggregate as determined by gel filtration. These data indicate that PFK is not an integral protein of the membrane, for such proteins tend to aggregate and lose activity in aqueous solution. Release of the enzyme by high ionic strength suggests that PFK is a peripheral enzyme of the membrane, one bound by electrostatic interactions.

Solubilized PFK (Triton-solubilized and NaCl-solubilized) has an approximate molecular weight of 300,000 daltons as determined by gel filtration.

Both the membrane-bound enzyme and the Triton-solubilized enzyme exhibit maximal activity over a wide pH range (7.4-8.5). The membrane-bound enzyme responds more drastically to assay in low pH than does the solubilized enzyme. Incubation of the particulate enzyme at low pH and subsequent assay at pH 7.8 revealed that the enzyme is irreversibly inactivated at the lower pH. Solubilized PFK does not exhibit this denaturation.

2. An in vitro ATP-dependent inactivation of membrane-bound activity. Incubation of a membrane pellet containing PFK activity at 30 C in the presence of NaF, MgATP, and the 100,000 x g supernatant of a crude extract results in the inactivation of enzyme activity. Absence of any one component of the inactivation mixture fails to decrease enzyme activity. The rate of inactivation is dependent on the concentration of MgATP as well as on the concentration of the supernatant.

Neither the Triton-solubilized enzyme nor the NaCl-solubilized enzyme is inactivated when incubated in the presence of MgATP, NaF, and the crude supernatant. Inclusion of a membrane pellet in the above incubation mixture does not promote inactivation of solubilized activity.

The supernatant factor needed for inactivation of membrane-bound PFK is heat labile, nondialysable, acid precipitable, and $(NH_4)_2SO_4$ precipitable. It has been partially purified by protamine sulfate precipitation, $(NH_4)_2SO_4$ precipitation, DE-52 column chromatography, and gel filtration.

MgGTP can replace MgATP in the inactivation mixture, whereas MgUTP and MgCTP cannot. AMP had no effect on activity of PFK in the presence of $MgCl_2$, NaF, and a dialyzed supernatant, but ADP did decrease activity under these conditions. Adenosine, inorganic phosphate, or pyrophosphate did not replace the requirement for ATP. Adenosine-3',5' cyclic phosphate did not affect activity under these conditions, nor did it affect the rate of inactivation in the presence of ATP.

Addition of the ATP analogue adenyl imidodiphosphate, AMP-P(NH)P, to the inactivation mixture in the place of ATP resulted in loss of PFK activity. Incubation with α,β-methylene ATP, $AMP(CH_2)PP$, did not decrease activity.

To determine if a small molecule is being produced in the inactivation mixture which in turn inhibits PFK, the supernatant of an inactivation mixture was filtered through an Amicon filter. Upon incubation of the filtrate with a membrane pellet, no loss of PFK activity was detected. To determine if a soluble enzyme was being modified which subsequently modified PFK, a dialyzed mixture of crude supernatant, MgATP, and NaF was incubated with a membrane pellet. PFK activity did not change under these conditions.

Inactivation of membrane-bound PFK in the presence of NaF, crude supernatant, and Mg[γ^{32}P]ATP, followed by solubilization of the enzyme using Triton X-100 and subsequent gel filtration resulted in the elution of one major radioactive peak. This peak (approximate molecular weight, 90,000 daltons) did not coincide with the peak of PFK activity of a control, but did coincide with the radioactive peak when PFK was inactivated in the presence of Mg[2,8-^{3}H]ATP. Similar gel filtration of a solubilized control mixture consisting of a pellet and Mg[2,8-^{3}H]ATP, however, also revealed a radioactive peak having the same elution volume as above.

Cells were grown in medium containing phosphorus-32. Gel filtration of Triton-solubilized membrane pellets of these cells revealed one radioactive peak which coincided with the radioactive peak obtained after incubation of membranes with Mg[2,8-^{3}H]ATP or of membranes, NaF, crude supernatant, and Mg[γ^{32}P]ATP. No radioactivity was associated with PFK activity.

Inactive PFK cannot be reactivated upon removal of ATP and NaF by dialysis of the inactivation mixture, by gel filtration of solubilized membrane proteins following inactivation of PFK, or by washing the membrane fraction following PFK inactivation. Also, incubation of inactivated PFK with acid phosphatase, alkaline phosphatase, or snake venom phosphodiesterase did not reactivate the enzyme.

Addition of crude supernatant to the dialyzed inactivation mixture as well as to the washed membrane fraction of an inactivated sample did not reactivate the enzyme. The activity of membrane-bound PFK was not increased upon incubation of membrane pellets in the presence or absence of crude supernatant. Addition of Ca^{2+} or Mg^{2+} to these incubation mixtures also failed to increase activity.

The inactivation of PFK was not prevented by the following protease inhibitors: phenylmethylsulfonyl fluoride, a serine protease inhibitor; tosyl lysine chloromethyl ketone, a trypsin inhibitor; or nitro-carboxy phenyl diphenylcarbamate, a chymotrypsin inhibitor.

Incubation of membrane pellets with high concentration of NH_4Cl or K_2HPO_4 before addition of NaF, MgATP, and crude supernatant, prevented loss of activity of PFK. Preincubation with AMP did not protect PFK from inactivation. It has been observed that crude supernatants of extracts prepared in potassium phosphate buffer generally have less inactivating activity than do those crude supernatants of extracts prepared in Tris-HCl buffer. Also, significant inactivation of PFK on membranes of Tris-HCl extracts is often seen upon incubation of the membranes with MgATP and NaF alone, whereas no decrease in activity is seen upon incubating membranes prepared in potassium phosphate buffer with MgATP and NaF.

Chelators enhance the loss of activity of PFK. Addition of EGTA or EDTA to mixtures of membrane pellets, MgATP, NaF, and crude supernatant results in activity loss greater than in the absence of chelator. As well,

EGTA or EDTA added to membranes incubated with only MgATP and NaF results in decreased PFK activity. Addition of Cd^{2+}, Mg^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, or Ni^{2+} to the inactivation mixture failed to overcome the loss of activity. Preliminary results indicate that Ca^{2+} or Mn^{2+} prevent the chelator-mediated decrease in activity.

Cholera toxin could not replace the requirement for NaF in the inactivation mixture. Cyclic AMP had no effect on PFK activity when added directly to membranes in the presence or absence of crude supernatant.

Discussion:

Since the rate of inactivation of membrane-bound PFK is dependent on the concentration of the crude supernatant or of the partially purified supernatant factor, it is apparent that the supernatant factor needed for the inactivation is an enzyme. Loss of membrane-bound PFK activity in the presence of MgATP, NaF, and the supernatant factor may be due to a proteolytic degradation of PFK, a ligand-induced conformational change of PFK from an active form to an inactive form, or a covalent modification of the enzyme.

Failure of three protease inhibitors to prevent the inactivation of PFK suggests that proteolysis of the enzyme is not occurring.

It is well documented that ATP is an inhibitor of PFK from most sources. Membrane-bound PFK from Tetrahymena, however, is not inhibited by high concentrations of ATP. If the loss in PFK activity described above is due to an ATP-induced conformational change, it is obvious that activity of a supernatant enzyme is involved in the loss of activity. Failure of the products of ATP hydrolysis, namely adenosine, AMP, inorganic phosphate, or pyrophosphate, to reduce activity suggests that hydrolysis of ATP is not responsible for the loss of PFK activity. The inactivation of PFK in the presence of ADP is probably due to conversion of ADP to ATP by endogenous adenylate kinase activity. Failure of the filtrate of an inactivation mixture to alter PFK activity suggests that the loss of PFK activity is not due to the production of other small molecules.

The finding that removal of ATP and its hydrolytic products by dialysis or gel filtration fails to reactivate the enzyme is further evidence against an allosteric inhibition of membrane-bound PFK. It is possible, however, that ATP is tightly bound to the enzyme and not removed by these methods. Absence of radioactivity associated with PFK activity upon using ^{32}P-labeled ATP or ^{3}H-labeled ATP indicates that ATP is not bound to the undissociated enzyme. It is possible that ATP or another molecule induces a conformational change in PFK which is not reversed upon gel filtration.

Membrane-bound PFK may be covalently modified in the presence of NaF, MgATP, and the supernatant enzyme. Failure to reactivate the enzyme upon removal of possible ligands strengthens this possibility. Absence of labeled, active enzyme upon growth of cells in phosphorus-32 suggests that

active PFK is not phosphorylated. If this is the case, inactivation of PFK is not due to dephosphorylation of the enzyme. Lack of correlation of either ^{32}P-activity or ^{3}H-activity with enzyme activity following incubation with Mg[$\gamma^{32}P$]ATP or Mg[2,8-^{3}H]ATP, respectively, as determined by gel filtration, indicates that the undissociated enzyme is neither phosphorylated nor adenylylated. The overlapping peaks of ^{32}P- and ^{3}H-activity indicate that ATP is binding very tightly to a component of the mixture. The appearance of a radioactive peak following incubation of a pellet and labeled ATP indicates that ATP is binding very tightly to a membrane component. A possible role of this binding in the inactivation of membrane-bound PFK is presently being investigated.

If the loss of PFK activity is dependent on the hydrolysis of ATP, it is suggested from the experiments using ATP analogues that it is the α,β-anhydride bond of ATP that is being cleaved. Failure of cholera toxin to replace the fluoride requirement and lack of inactivation of enzyme activity by the addition of cyclic AMP suggests that adenyl cyclase activity is not responsible for PFK inactivation. As discussed, it is improbable that PFK is being inactivated by adenylylation, and unlikely that products of ATP hydrolysis are inhibiting activity. If the differences in the analogue effects are not due to specific bond cleavage, perhaps the differences reflect binding potential of the analogues to the enzyme.

The finding that solubilized PFK cannot be inactivated when incubated with NaF, MgATP, and crude supernatant may indicate a direct role of the membrane in the inactivation of the membrane-bound enzyme. Failure of the addition of a membrane pellet to the above mixture to inactivate solubilized PFK further suggests that proximity to membrane components is needed for inactivation. The irreversible inactivation of membrane-bound enzyme and not of solubilized enzyme at low pH indicates that the conformation of the membrane-bound enzyme and that of the solubilized enzyme may differ. If so, this difference may contribute to lack of inactivation of the solubilized enzyme.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 00217-02 LB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Control of in vitro Assembly of Microtubules

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

PI:	Donita L. Garland	Staff Fellow	LB NHLBI
OTHER:	Earl R. Stadtman	Chief, Lab. of Biochem.	LB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
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SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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 binding mechanism for colchicine and tubulin was elucidated from kinetic studies. The mechanism is a two step binding process consisting of an initial rapid equilibrium followed by a rate limiting conformational step. Rate constants for all steps were determined.

Modification of tubulin by membrane components change the interaction of colchicine with tubulin. The nature of the modification is not yet known.

In vitro polymerization in crude supernatant was inhibited by sodium fluoride, microsomal fraction, GMP, AMP, and to a lesser extent by CMP. These effects were not observed with purified tubulin. GMP does not inhibit the ATP induced polymerization. The inhibition is not due to chelation of Mg^{++} nor to competition for GTP at the exchangeable nucleotide site on tubulin.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives:

I. The formation of microtubules *in vitro* is now possible using either supernatant prepared from brain homogenates or purified tubulin. While it appears tau protein is the only additional protein required for assembly of purified tubulin, it seems likely that other proteins are important for control of assembly-disassembly of microtubules in the cell.

There is considerable evidence indicating there is a phosphorylation-dephosphorylation cycle for tubulin, however, the role it has is not known. For this reason the effect of sodium fluoride, a known modifier of phosphate transfer enzymes, on *in vitro* polymerization of tubulin was studied.

II. Colchicine prevents the assembly and causes the disassembly of microtubules by its unique binding to tubulin. Kinetic and thermodynamic data obtained previously using radiolabeling techniques were inconsistent with a simple bimolecular reaction scheme. Kinetic studies were continued to obtain a precise binding mechanism and the rate constants.

Major Findings:

I. Sodium fluoride inhibits the *in vitro* assembly of microtubules in crude supernatants but not purified tubulin and only to a small extent in crude supernatants that have been gel filtered. Incubation of crude supernatant with sodium fluoride resulted in an increase in the level of GMP. It was found that GTP dependent *in vitro* assembly of microtubules was inhibited by GMP, AMP, CMP to a lesser extent, but not by c-AMP. The inhibition was not due to chelation of Mg^{++} nor to competition for GTP to the exchangeable site on tubulin. GMP does not inhibit the ATP induced polymerization at concentrations that inhibit the GTP induced polymerization. Therefore it is unlikely that the inhibition is due to an effect of a GTP regenerating system. The effects of GMP on the assembly of partially purified tubulin vary with the preparation which may reflect differences in the complement of contaminating proteins. The ATP induced assembly of partially purified tubulin was inhibited by high concentrations of GMP and greater than 1 mM Mg^{++} and CrAMP, but not by AMPPNP, diadenosine pentaphosphate or sodium fluoride.

II. The binding mechanism for colchicine & tubulin was elucidated from kinetic studies. Binding was determined using radiolabeled colchicine and a fluorometric technique. Rates of binding show a non linear dependence on colchicine and protein concentrations. This is consistent with a two-step binding scheme consisting of an initial rapid equilibrium, $K = 3.8 \times 10^{3} M^{-1}$ followed by a rate limiting conformation, $k_2 = 2.8 \times 10^{-2}$ sec $^{-1}$. Tubulin is modified by a component of the brain particulate fraction that is released by gentle sonication and detergents. This results in a 3-5 fold increase in the rate of dissociation of the colchicine-tubulin complex (NOT denaturation of the tubulin) and no apparent change in the rate of binding. The nature of the modification is not known.

Relevance to Medicine:

Microtubules are required for development of cell shape, motility, mitosis and all cases in which intracellular particle movement occurs, for example axonal transport and secretion. The requirement for microtubules in mitosis makes them important for any cell reproduction including malignant cell growth and wound healing. Thus the importance of studying the function and regulation of microtubules is obvious.

Colchicine prevents assembly and causes disassembly of microtubules *in vivo*. Even though the exact mechanism of this action of colchicine is not yet known, colchicine has found wide use in studying the role of microtubules in cellular function. Colchicine is also used as an antitumor agent and is a classic treatment for gout. A thorough understanding of the interaction between colchicine and tubulin should provide insight into the mechanism of polymerization and microtubule function.

There are a few known cases in which it appears a defect in control may either primarily or secondarily affect microtubule function thereby contributing to disease: insulin secretion in diabetes, lung dysfunction and infertility in cases of non motile cilia and flagella, abnormal microtubules in Alzheimers disease and impaired function in Chediak-Higashi syndrome.

Proposed Course of Action:

Efforts will be continued to determine the mechanism of inhibition of assembly of microtubules in crude supernatant by the microsomal fraction, sodium fluoride, GMP and AMP. It is necessary to determine if sodium fluoride has other effects unrelated to the increase in GMP concentration that alter the state of assembly-disassembly.

Publications:

Garland, D.L. (1977) Fed. Proc. 36, 899. Evidence for Colchicine Induced Protein Conformational Change in Tubulin.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00218-02 LB

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Formate Dehydrogenase from Methanococcus vannielii.

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

PI:	Jay B. Jones	Microbiologist	LB NHLBI
OTHER:	Thressa C. Stadtman	Chief, Section on Intermediary Metabolism & Bioenergetics	LB NHLBI

COOPERATING UNITS (if any)

Blair Bowers, Ph.D. Laboratory of Cell Biology, NHLBI, NIH

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SECTION

Section on Intermediary Metabolism and Bioenergetics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

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1.1	.2	.9

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Formate dehydrogenase from cell extracts of M. vannielii has been resolved into two forms by ammonium sulphate fractionation. Both forms, P-I and P-II, are oxygen sensitive, are stable at 60°C, have optimal catalytic activity at pH 9.1, and are inhibited by cyanide. P-I has been purified to near homogeneity and has been shown to contain approximately 15 moles of iron and 2 moles of molybdenum per mole of enzyme. P-II contains selenium while P-I does not; P-II is absent in extracts of cells grown without selenite. SDS gel filtration chromatography shows P-I and P-II formate dehydrogenase in identical positions perhaps suggesting that P-II exists as a complex of P-I and selenoprotein.

A low molecular weight yellow compound, F_{420}, has been isolated from extracts of M. vannielii. F_{420} shows a blue-green fluorescence in ultraviolet light and an absorption peak at 420 nm when oxidized; the color, fluorescence and 420 nm peak disappear upon reduction. Partially purified extracts show formate dependent NADP reduction with an absolute requirement for F_{420}. P-I but not P-II shows this identical formate-NADP oxidoreductase activity.

75

Project Description:

Objectives: Studies are currently under way to characterize the components of formate dehydrogenase in the methanogenic anaerobe Methanococcus vannielii and to determine how this enzyme functions in providing reducing equivalents for reduction of carbon dioxide to methane. Emphasis is being placed on studying the trace element content of formate dehydrogenase and how these trace elements (i.e. selenium molybdenum and iron) function during catalysis.

Major Findings:

During ammonium sulfate fractionation of M. vannielii extracts, two stable forms of formate dehydrogenase are found. These two forms, P-I and P-II, are purified separately.

I. Factor 420: A highly fluorescent dialyzable moiety has been isolated from extracts of M. vannielii. This factor, F_{420}, has a conspicuous absorption peak at 420 nm and a fluorescence emission peak at 470 nm when excited at 425 nm in 0.1 N NaOH. F_{420} loses its absorption at 420 nm when reduced with borohydride or dithionite and regains it upon reoxidation in air. Both P-I and P-II reduce F_{420}.

II. F_{420} dependent formate-NADP oxidoreductase: Partially purified extracts of M. vannielii have been shown to reduce NADP but not NAD in the presence of formate and F_{420}. This formate dependent NADP reduction has a pH optimum of 7, a temperature optimum at 45°C, and an absolute requirement for F_{420}. P-I but not P-II exhibits this enzymic activity.

III. Purification of P-I: This formate dehydrogenase form has a molecular weight of approximately 200,000. Trace metal analysis on a nearly homogeneous preparation shows approximately 15 moles of iron, two moles of molybdenum, and no selenium per mole of enzyme. The oxygen inactivation of this form is partially reversed by adding dithionite and iron to oxygen inactivated enzyme.

IV. Purification of P-II: This formate dehydrogenase form has a molecular weight in excess of 500,000, has pH and temperature optima similar to P-I and contains selenium. P-II is absent in extracts of cells grown without added selenite. In extracts of cells grown with 100 µM ^{185}W tungstate, P-II is enhanced and P-I disappears; additionally, ^{185}W is copurified with P-II. When P-II is run over an A 1.5 M column in 0.25% SDS, a formate dehydrogenase peak appears at the position where P-I is found. These data may suggest that P-II exists as a complex of P-I, selenoprotein, and other protein components. Tungsten may serve to stabilize this complex.

Proposed course of action:

The selenium containing components of P-II will be isolated by SDS column chromatography of ^{75}Se labeled formate dehydrogenase. This component will be

hydrolyzed and subjected to amino acid analysis to determine if selenocysteine is the form of selenium in the enzyme. Trace metal analysis of this and other components will be done by atomic absorption spectroscopy in order to determine the sites of incorporation of Mo, W, and Fe.

The apoprotein of F_{420} will be purified using the NADP reductase spectrophotometric assay. The nature of the binding of F_{420} to apoprotein will be examined.

The possibility that oxidation and loss of iron may be the basis for the oxygen inactivation of formate dehydrogenase will be explored by using iron chelating resins and atomic absorption analysis to correlate loss of iron to loss of enzymic activity. The form of iron in P-I will also be examined.

Publications:

Jay B. Jones and T.C. Stadtman. Methanococcus vannielii: Growth and Metabolism of Formate. In "Microbial Production and Utilization of Gases" E. Goltze KG, Göttingen, p. 199-205 (1976).

T.C. Stadtman, J.E. Cone, R. Martin del Rio, J.B. Jones and B. Seto. Selenoenzymes of Bacteria. Proc. of Symp. on Selenium-Tellurium in the Environment. Univ. of Notre Dame, Notre Dame, Ind. p. 226-233 (1977).

J.B. Jones and T.C. Stadtman. Methanococcus vannielii: Culture and Effects of Selenium and Tungsten on Growth. J. Bacteriol. 130, 1404-1406 (1977).

J.B. Jones, Blair Bowers & T.C. Stadtman. Methanococcus vannielii: Ultrastructure and Sensitivity to Detergents and Antibiotics. J. Bacteriol. 130, 1357-1363 (1977).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HO 00219-02 LB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Effect of glucose starvation on the NADPH-dependent glutamate dehydrogenase of yeast.

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

PI:	Maria J. Mazón	Visiting Scientist	LB NHLBI
OTHER:	E. R. Stadtman	Chief, Lab. of Biochem.	LB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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1	1	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

In a search for a system with which to study the mechanisms of protein degradation, the specific activity of the NADPH-dependent glutamate dehydrogenase of Saccharomyces cerevisiae was found to drop to 30% of its initial value upon glucose starvation of glucose grown cells. The modification leading to the loss in specific activity is irreversible as demonstrated by the necessity of "de novo" protein synthesis for recovery of the activity.

The loss of specific activity has been shown to be inhibited by energy uncouplers, inhibitors of protein synthesis and a serine protease inhibitor.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To study the conditions required "in vivo" for the inactivation of the NADPH-dependent glutamate dehydrogenase and to find an "in vitro" system which is able to inactivate the enzyme.

Major Findings:

A loss in the specific activity of the NADPH-dependent glutamate dehydrogenase was found when yeasts grown on minimal medium containing glucose as the sole carbon source, were transferred to a medium lacking glucose. Age of the cells at the time of starvation and the presence or absence of vitamins or trace elements in the starvation medium did not show any effect on the extent of inactivation.

When glucose was added to the medium, after inactivation, the activity was recovered but the reappearance of the enzyme was prevented in the presence of cycloheximide, indicating that the process leading to the loss of catalytic activity was irreversible. The loss of specific activity was blocked when 2,4-dinitrophenol, sodium azide or iodoacetic acid were added at the onset of glucose starvation. The presence of cycloheximide prevented the inactivation as well.

These findings seem to indicate that when the yeasts are subjected to glucose starvation, the synthesis of a protein takes place which is involved in the degradation of the NADPH-dependent glutamate dehydrogenase.

It was also found, that when phenylmethylsulfonyl fluoride, an inhibitor of serine proteases, was added to the starvation medium, the inactivation of the enzyme was partially prevented. This result is consistent with the participation of a protease or proteases in the inactivation process.

However, extraction of the starved cells, at different times during the inactivation process, immediately stopped the inactivation indicating that some important factors are needed in order to continue the inactivation "in vitro". Mixtures of extracts from starved and control cells showed to be very stable. The extracts from starved cells did not recover the activity after dialysis nor showed any inhibitory effect on the activity of extracts from control cells, indicating that the loss of activity is not due to the appearance of a low molecular weight dissociable inhibitor under the glucose starvation conditions.

When a yeast autolysate was incubated at an acidic pH in order to destroy the protease inhibitors known to be present in yeasts, a fraction enriched in proteolytic activity was obtained. This fraction was able to inactivate the NADPH-dependent glutamate in extracts and this inactivation was totally inhibited by phenylmethylsulfonyl fluoride.

At present we are trying to get the inactivation in cells that have been made permeable to small molecules by treatment with either toluene or basic molecules such as protamine sulfate, cytochrome C or chitosan.

Proposed course of action:

To establish the conditions needed to inactivate the NADPH-dependent glutamate dehydrogenase in permeabilized cells and, eventually in protoplasts.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00221-01 LB

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Control of Glutamate Metabolism in Yeast

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

PI:	Brian A. Hemmings	Visiting Fellow	LB NHLBI
OTHER:	E. R. Stadtman	Chief, Lab. of Biochem.	LB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Section on Enzymes

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Earlier in vivo studies (Hemmings and Sims, Eur. J. Biochem., in press) suggested that the NAD-dependent glutamate dehydrogenase from the food yeast, Candida utilis, existed as two interconvertible forms, which could be rapidly inactivated and reactivated. The aim of this project is to elucidate the biochemical mechanism of interconversion. To date, the active form of the enzyme has been purified by affinity chromatography and conventional purification techniques: antibodies have been raised against this protein in sheep. We intend to further characterize and purify the active and inactive forms of the NAD-GDH and the proteins responsible for the interconversion.

Project Description:

Glutamic acid occupies a central position in the nitrogen metabolism of yeast and other microorganisms; therefore it is important to understand the controls exerted on the enzymes involved in its synthesis and degradation. Glutamate synthesis in fungi is believed to be mediated by the NADP-dependent glutamate dehydrogenase and the NAD-dependent glutamate dehydrogenase is thought to be primarily responsible for its degradation.

Growth of C. utilis on glutamate, as nitrogen or nitrogen, carbon and energy source, leads to extensive derepression of NAD-GDH activity. Transfer of glutamate grown yeast to starvation medium or transfer to NH_4^+ containing medium was found to result in rapid enzyme inactivation, associated with changes in the properties of the NAD-GDH. Addition of glutamate to starvation medium (cells previously grown on glutamate) promoted rapid and complete restoration of enzyme activity independent of protein synthesis. These data suggested that the NAD-GDH from yeast may be controlled by some kind of covalent modification. Therefore the objective of this investigation is to elucidate the mechanism for the rapid modulation of enzyme activity.

Major Findings:

(1) Purification of the active NAD-GDH. Yeast were grown on glutamate as sole nitrogen, carbon and energy source, which leads to complete derepression of enzyme synthesis, and were used as starting material for purification.

The enzyme has been purified essentially to homogeneity by ammonium sulfate fractionation, DEAE-cellulose chromatography, affinity chromatography on Cibacron Blue F3GA-agarose and gel filtration on Sephacryl-S200. However, the purified protein contains small amounts of contaminating proteins as visualized by SDS-polyacrylamide gel electrophoresis. These two contaminating proteins probably result from proteolysis of NAD-GDH by trace amounts of proteases which copurify up to the gel filtration step. Addition of mM phenylmethylsulfonyl fluoride (protease inhibitor) to the purification buffers greatly reduces the amount of the two protein contaminants.

From SDS-polyacrylamide gel electrophoresis the enzyme was shown to have a subunit molecular weight of about 110,000. The NAD-GDH isolated from Neurospora crassa has a similar subunit size; both enzymes appear to be considerably different from other GDH's isolated from eukaryotic cells due to subunit size.

Attempts have been made to purify the inactive form of the NAD-GDH. Using small amounts of yeast (starved for glutamate) it is possible to obtain maximally inactivated GDH, however, with large scale preparations the enzyme was found to spontaneously reactivate. Methods are being sought which stabilize the enzyme in the inactive state so that it can be purified.

(2) Antibody preparation. Antibodies have been raised in sheep against the purified active NAD-GDH. From analysis by Ouchterlony double diffusion the antiserum appears to be monospecific. The antibody preparation is capable of inhibiting and precipitating active and inactive NAD-GDH from crude cell free extracts. Experiments are in progress to demonstrate that antigen (NAD-GDH) levels remain constant during enzyme inactivation, adding further evidence to the hypothesis that inactivation involves a covalent modification.

Proposed Course of Action:

We intend to purify the inactive NAD-GDH and characterize both forms of the enzyme. Preliminary experiments have shown that reactivation of the inactive enzyme (from crude cell free extracts and the partially purified form) in vitro is possible. Once the inactive NAD-GDH can be obtained in sufficient amounts, we propose to use this protein preparation as substrate for isolation of the protein(s) responsible for reactivation.

Using the antibody specific for NAD-GDH, we will use immunoprecipitation rechniques to perform a one-step purification of the enzyme. This method will be used in conjunction with radioactive labeling experiments using ^{32}P. By use of these methods it is hoped to demonstrate whether the enzyme activity is modulated by phosphorylation or adenylylation during inactivation.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00222-01 LB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Regulation of glutamine synthetase of A. vinelandii.

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

PI:	Joachim Siedel	Visiting Fellow	LB NHLBI
OTHER:	E. R. Stadtman	Chief, Lab. of Biochem.	LB NHLBI

COOPERATING UNITS (if any)

none

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.8	

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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)

Growth of A. vinelandii in a modified Burk's medium containing glutamate leads to high cellular levels of glutamine synthetase in the late stationary phase. The enzyme has been purified to homogeneity with a purification method involvin a selective precipitation with Zn in the presence of Mg. The enzyme has been examined with regard to a possible regulation by an adenylylation-reaction.

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Project Description:

Objectives: (1) To determine growth conditions for cultures of Azobacter vinelandii leading to high cellular levels of glutamine synthetase. (2) To develop a method for the purification of this enzyme from A. vinelandii. (3) To study whether the glutamine synthetase of A. vinelandii can be modified by an adenylylation-deadenylylation reaction similar to that of the E. coli glutamine synthetase.

Major Findings:

(1) Out of all growth conditions tested, cells with the highest specific activity of glutamine synthetase (up to 2.5 units/mg protein as measured by the Mn-dependent transferase assay with crude cell extracts) were obtained from the late stationary phase 4-5 days after the onset of growth in a modified Burk's medium containing sucrose as carbon source and 10 mM L-glutamate. Crude extracts of cells grown in a similar medium without any combined nitrogen contained about 1.5 units/mg, whereas growth on 10 mM NH_4Cl led to a specific activity of about 0.5-1.0 units/mg. Under all circumstances, the specific activity of the glutamine synthetase in the Mn-dependent transferase assay was lowest during the logarithmic phase of cell growth.

(2) As is the case with the E. coli enzyme, the glutamine synthetase of A. vinelandii can be precipitated rather selectively from crude cell extracts in the presence of Mg and low concentrations of Zn at room temperature. However, solubilization of the precipitated enzyme is possible only with buffers containing EDTA, which also leads to solubilization of most of the co-precipitated impurities. More selectivity, but a distinctly reduced yield can be achieved using buffers with high concentrations of Mg for solubilization.

In order to make the Zn-precipitation as efficient as possible, the enzyme is partially purified from the crude extract with a purity of about 50% prior to the Zinc treatment. This is achieved by the following steps: (a) streptomycin sulphate treatment of the crude extracts, (b) ammonium sulphate precipitation, (c) acid precipitation, (d) chromatography on DEAE-cellulose, and (e) gel-filtration on Bio-Gel. After these steps the enzyme can be precipitated with Zn, often in the form of small crystal-like aggregates; however, these aggregates are not stable, especially at cold temperatures. Moreover, prolonged contact of the enzyme with Zn leads to a complete inactivation.

SDS-disc gel electrophoresis of the purified enzyme leads to one single band of protein with a molecular weight of about 59,000 daltons (preliminary determination).

(3) Due to the relatively low yield of pure enzyme (~5%) a definite answer to the question whether this enzyme undergoes a modification by an adenylylation-deadenylylation reaction still remains open. However, many observations on the behavior of the enzyme under certain conditions indicate

a close similarity between the glutamine synthetases of E. coli and A. vinelandii. Among these, the most important are: (a) addition of 10 mM NH_4^+ to cultures of cells grown on glutamate immediately before harvesting leads to a 3-fold decrease of the Mg-dependent transferase activity as compared to the extract of cells untreated with NH_4^+. (b) Treatment with snake venom phosphodiesterase of the purified enzyme from cells grown on glutamate and NH_4^+ leads to a two- to three-fold increase in the Mg-dependent transferase activity.

Proposed Course of Research:

We will try to further improve the yield of the purification procedure for the enzyme in order to be able to identify any attached nucleotide by its isolation after treatment of the enzyme with snake venom phosphodiesterase and characterization with thin layer chromatography. Further evidence could be obtained by growth of the bacteria on a medium containing ^{32}P, followed by determination of any incorporation of radioactive label into the enzyme under conditions leading to an adenylylation in the case of the E. coli enzyme.

Publications: none

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 00223-01 LB

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Purification and some properties of large components of glycine reductase.

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

PI:	Hidehiko Tanaka	Staff Fellow	LB NHLBI
OTHER:	Thressa C. Stadtman	Chief, Section on Intermediary Metabolism & Bioenergetics	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, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.9	0.9	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The large molecular weight components of the clostridial glycine reductase system are separated by rechromatography on DEAE into two protein components, protein B and fraction C, which separately are enzymatically inactive. However, they combine to form the enzymatically active complex in the presence of protein A. Protein B was purified to a high degree, but fraction C still is heterogeneous.

Protein B displays a high sensitivity to carbonyl reagents. Based on this observation, an independent assay for protein B that measures the release of tritium from 2-^{3}H-glycine to water or the incorporation of tritium from $^{3}H_2O$ into glycine was developed. The formation of ammonia or ATP required the participation of all three protein components.

Major Findings:

(1) Purification of the large molecular weight components of glycine reductase system: The large molecular weight components were separated into two protein components, called protein B and fraction C. Separately, these components are catalytically inactive, however, they combine to form the enzymatically active complex. During the initial steps of purification including the first DEAE-step, these two components were purified together. After detergent treatment, they were separated by rechromatography on DEAE and from this point, each of them was purified by separate procedures. The final preparation of protein B exhibited three minor bands besides one major band as determined by polyacrylamide electrophoresis. Fraction C is heterogeneous.

(2) Inactivation of protein B by carbonyl reagents and protein B catalyzed exchange reaction: When each of the protein components was separately treated with borohydride or hydroxylamine, and then various combinations of treated and untreated proteins were assayed for glycine reductase activity, protein B was found to be completely inactivated. Treatment of fraction C with hydroxylamine resulted in partial destruction of catalytic activity, but borohydride resulted in only a slight inactivation. These findings indicated that a carbonyl group might be on protein B. Addition of pyridoxal phosphate to protein B treated with hydroxylamine failed to reconstitute active protein B.

Based on this observation, an independent assay for protein B that measures the release of tritium from 2-^{3}H-glycine to water or the incorporation of tritium from $^{3}H_2O$ into glycine was developed. These two exchange reactions were catalyzed by protein B alone and were employed as the assay procedure for protein B purification.

(3) Effect of salts: The catalytic activity of the glycine reductase multienzyme complex is affected by various salts. Especially, the effect of anion seems to be greater than cation; activation by sulfate ion and inhibition by chloride ion. This phenomenon may be related to conformational effects required for interaction of the protein components of the system.

(4) Formation of another product, ammonia, from glycine required all components, protein A, B and fraction C. Ammonia was not formed in the absence of added DTT as electron donor or phosphate-adenylate system even if three protein components exist in the reaction mixutre. Formation of ATP required the participation of three components, alone each protein or various combinations of two proteins is completely inactive.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00224-01 LB

PERIOD COVERED
July 1, 1976 to September 30, 1977

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

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

PI: Charles Y. Huang	Expert	LB NHLBI
OTHER: E. R. Stadtman	Chief, Laboratory Biochemistry	LB NHLBI

COOPERATING UNITS (if any)
Seymour Kaufman and Norman Chang, Laboratory of Neurochemistry, NIMH

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
National Heart, Lung and Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

(1) Development and application of kinetic methods, including a constant-ratio alternative substrate approach for differentiating enzyme mechanisms, an improved procedure for deriving steady state and isotope exchange equations, and application of these methods to the study of the flip-flop model of E. coli alkaline phosphatase and the reaction mechanism of rat liver phenylalanine hydroxylase.

(2) Purification and characterization of two key enzymes in the cascade control system of E. coli glutamine synthetase, the uridylyl-transferase and the uridylyl-removing enzyme.

(3) Other projects to be pursued: interactions of cyclic nucleotide phosphodiesterase, its protein modulator, and the modulator binding protein; theorectical and experimental studies of multiple inhibition in enzyme-catalyzed multisubstrate reactions.

Project Description:

Objectives:

(1) Development and refinement of kinetic methods which provide more effective means for the studies of enzyme mechanisms and regulation, particularly for enzyme systems in which alternative substrates are often the best tools, such as cyclic nucleotides, protein substrates, and phosphoproteins in the protein kinases, phosphatases and cyclic nucleotide phosphodiesterase catalyzed reactions.

(2) Understanding of the properties and interactions of proteins involved in the cascade control of enzymes interconvertible by covalent modification,particularly the uridyltransferase (UTase) and uridylyl-removing enzyme (UR) in the E. coli glutamine synthetase system.

Major Findings:

(1) A new kinetic approach utilizing alternative substrates as a tool for differentiating enzyme mechanisms has been developed. The main features of this technique are the diagnostic graphical plots obtained by keeping the alternative substrates at several constant ratios while measuring the common product formed in the reaction. These patterns are different for various mechanisms, thus permitting one to select a kinetic model. Although alternative substrates have been used as a kinetic probe in the past, the usual approach is to keep the alternative substrate at a constant level. In this conventional method, when a unique product is measured, in a bireactant system, for instance, the resultant reciprocal patterns are invariably competitive with one and noncompetitive with the other substrate. Its only utility is the confirmation of an ordered reaction pathway when nonlinear plots are observed. When the common product is measured, on the other hand, the resultant patterns are often nonlinear, making it ineffective for data analysis purposes. The newly developed method overcomes these shortcomings, making it a truly useful kinetic tool. It has been applied to test a flip-flop model which is proposed for alkaline phosphatase to explain the function of subunits in an enzyme displaying highly negative cooperativity (see report by Judith R. Bale and P. B. Chock for detail). It has also been used to study the reaction mechanism of phenylalanine hydroxylase. Details of this study is described in a later section.

(2) An improved general method for deriving steady state and isotope exchange equations has been developed. This method includes two general rules which eliminates the redundant terms that appear in the method of Fromm (Biochem. Biophys. Rsch Comm. 40, 692, (1970)) and provides a consecutive branching method for eliminating the closed-loop terms in complex mechanisms. A procedure applying this method to the derivation of isotope exchange equations based on the method of Cleland (Ann. Rev. Biochem., 36, 77 (1967)) contains the following improvements: (a) elimination of unnecessary branches (King-Altuan branches), (b) use of compressed diagrams. This improved procedure greatly simplifies the mathematical manipulations involved in the derivation of rate equations.

(3) In collaboration with Dr. Seymour Kaufman's group, Laboratory of Neurochemistry, NIMH, the reaction of rat liver phenylalanine hydroxylase has been studied. The kinetic behavior of this enzyme is of interest for a number of reasons. First, the mechanism of action of pteridin cofactor-dependent hydroxylases has not been investigated in detail. Secondly, the required activation of molecular oxygen in monooxygenase-catalyzed reactions has prompted chemists in the past to propose a ping-pong type mechanism which would be consistent with the "oxene" concept advocated by Hamilton (in O. Hayaishi ed., Molecular Mechanisms of Oxygen Activation, Academic Press, New York, 1974). Kinetic experiments with phenylalanine hydroxylase are difficult because the variation of oxygen at different constant tensions is hard to achieve. In addition, the irreversible nature of the hydroxylation reaction precludes the use of equilibrium isotope exchange technique. Product inhibition studies are further limited by the instability of one of the products, quinonoid dihydropterin. Thus, alternative substrates, 6-methylpterin and fluoro-phenylalanine were utilized to deduce the mechanism by the constant-ratio approach described in (1). The mechanism was found to be rapid equilibrium random, demonstrating that all three substrate must bind to the active site before the oxygen can be activated.

(4) The UTase and UR are key components in the cascade control system of E. coli glutamine synthetase. Characterization of these enzymes, however, has been hindered by the lack of preparations in purified form. Attempts were made to isolate these proteins by methods that have not been tried by previous workers. It was found that precipitation at pH 5.2 led to a seven-fold purification of UTase activity over the crude extract after removal of nucleic acids with 1% streptomycin sulfate. It was also found that on storage, UTase coprecipitated with unknown proteins which could not be resolubilized. This may account for the previous observations that on passing over DEAE-cellulose column, 70-100% of enzyme activity was lost--possibly due to precipitation on the column. When the resuspended pH 5.2 fraction was re-precipitated by 30% saturation of ammonium sulfate, massive precipitation of protein also occurred upon storage, but the UTase activity remained in solution. As a result, a four-fold purification was achieved by centrifugation to eliminate the precipitant. Further purification will be carried out in collaboration with Dr. S. G. Rhee.

When crude protein preparations were pulse-heated to 60°, the UTase activity was selectively destroyed while the UR retained 90% of its original activity. This experiment, together with the observation that pH 5.2 precipitation resulted in differential distribution of UTase and UR activities in the precipitate and the supernatant, seems to indicate that these two activities may be individually or preferentially destroyed under various conditions. Alternatively, it could mean that they are separate proteins. Experiments with mutants from Salmonella, which are void of UTase activity, however, yielded mixed results. The UR activity was only observed occasionally. A definitive answer will have to await the isolation of the enzymes.

For certain crude preparations containing UR activity from various sources (E. coli, Pseudomonas, and Salmonella), activation was observed in the presence of ATP and α-ketoglutarate. The effect of ATP and α-ketoglutarate was higher than the additive of ATP and α-ketoglutarate alone. In addition, the UR after heat treatment showed absolute requirement of ATP and α-ketoglutarate for catalysis. Whether these compounds are true effectors for UR, however, is uncertain because of erratic reproducibility of such activation. It might depend on the growth condition of the bacteria or is simply an artifact due to the interference of other enzymes in the crude extract.

The assay procedures for UTase and UR, utilizing the glutamine synthetase cascade system, have been modified so that their activities can be more accurately monitored in the early stages of purification. The UTase activity is measured as the difference in the presence and absence of UTP to eliminate the contributions from endogenous P_{IID} and unadenylylated glutamine synthetase. In the UR assay, the control contains CMP without added Mn++, whereas the assay samples contain Mn++ but no CMP. The strong inhibitory effect of CMP on UR is utilized to eliminate the residual UR activity due to metal ions present in the crude extract. The validity of these modified procedures has been verified by Dr. S. G. Rhee using tritiated P_{IID} as the substrate.

Significance to Biomedical Research: To gain knowledge of the fundamental principles involved in the regulation of metabolism through the interaction of enzymes and effectors and to develop useful tools for the pursuit of this goal.

Proposed Course of Research:

(1) To purify and study the properties of UTase and UR.

(2) To develop theory relating to the effect of multiple inhibitors in multisubstrate reactions.

(3) Collaboration with Dr. P. B. Chock, Dr. C. B. Klee, Laboratory of Biochemistry, NCI, and Dr. J. H. Wang, Department of Biochemistry, Faculty of Medicine, University of Manitoba, Winnipeg, Canada, on the study of interactions of cyclic nucleotide phosphodiesterase, its modulator protein (activator), and the modulator binding protein (inhibitor of the phosphodiesterase activator).

Publications:

Huang, C. Y.: Constant-Ratio Alternative Substrate Approach for Differentiating Enzyme Mechanisms. Arch Biochem. Biophys., in press.

Bale, J., and Huang, C. Y.: Mechanism of E. coli Alkaline Phosphatase: Kinetic Evidence Contrary to the Flip-Flop Model. Fed. Proc. 36, 635, 1977.

Annual Report of the
Laboratory of Cell Biology
National Heart, Lung, and Blood Institute
July 1, 1976 to September 30, 1977

Progress in four areas of investigation in the Laboratory of Cell Biology will be discussed in this summary: muscle biochemistry, cell motility, microtubule assembly, and electron transport.

Muscle Biochemistry: Pre-steady state and steady state kinetic studies in this and other laboratories have led to the identification of a number of intermediates in the hydrolysis of ATP by skeletal muscle myosin and actomyosin. For example, the reaction scheme for hydrolysis of ATP by myosin can be written: M+ATP → M-ATP → M*ATP → M**ADP-Pi, where M, M*, and M** represent three different conformational states of myosin with fluorescent changes being associated with the M* and M** states, and where ATP and ADP-Pi represent myosin-bound substrate and products, respectively. The formation of the first myosin-product complex, M**ADP-Pi, is identical to the so-called initial burst of Pi formation, i.e., the initial release of Pi that occurs more rapidly than the steady state rate of Pi formation.

Tonomora has proposed that the two ATPase sites (one on each myosin subunit head) behave differently in this reaction scheme, i.e. that one head binds ATP slowly and reversibly and does not contribute to the initial burst while the other head binds ATP rapidly and irreversibly to cause the initial burst. According to this hypothesis, only 50% of the myosin heads should participate in the initial burst. This idea was tested directly in this laboratory by stopped-flow pre-steady state kinetic analysis using a three-syringe apparatus and the soluble myosin fragment S_1 as the enzyme. About 80% of the S_1 heads were found to bind ATP irreversibly and 70% of the bound ATP contributed to the initial burst. This is significantly more than the 50% predicted by Tonomora. Although still short of 100% the difference may be due to the presence of denatured enzyme.

As mentioned, the initial burst is defined as the rapid release of Pi before reaching the steady state rate. Work in this laboratory has now shown that the rates of fluorescence change and absorbance change of myosin S_1 and the rate of H^+ release all level off as the ATP concentration is increased. This is compatible with the idea that each of these changes is due to the conformational change in myosin that occurs when the ATP is irreversibly bound and hydrolyzed, i.e. all are reflections of the initial burst phenomenon.

In the reaction scheme shown above, there are two fluorescent states of myosin. Studies in this laboratory have now shown that the major portion of the change in fluorescence of S_1 occurs in the step M*-ATP → M**ADP-Pi and that this step is much slower than the prior step M-ATP → M*-ATP which accounts for less of the overall change in fluorescence.

When the actomyosin ATPase cycle is studied rather than the myosin ATPase cycle, there is a different rate-limiting step which is the transition of a refractory state of myosin (that cannot bind actin) to a non-refractory state of myosin (that can bind actin). The above data make it possible to postulate

that the transition from refractory to non-refractory myosin may correspond to the transition from M*-ATP to M**ADP-Pi. Studies of the rebinding of actin to myosin S_1 show that at 15°, as previously determined for 5°, the rate of rebinding of actin determined by pre-steady state stopped-flow kinetic studies is the same as the V_{max} of the actomyosin ATPase. This, of course, agrees with the earlier proposal that it is the refractory to non-refractory myosin transition that controls the overall rate of actomyosin ATPase.

The functioning form of actomyosin, *in vivo*, must be a ternary complex between actin, myosin and ATP which converts to the ternary complex of actin, myosin, ADP. The ternary complex with ADP has previously been shown to exist both *in situ* and *in vitro*, but the ternary complex involving ATP is too transitory to detect directly. Using myosin S_1 and a non-hydrolyzable analogue of ATP, AMP-PNP, which is known to allow formation of the rigor complex *in vivo* (the rigor complex is presumably the ternary complex), it was hoped to be able to gain information that would be applicable to the natural ternary complex with ATP. It has been found that, at low ionic strength, actin binds to the binary complex of S_1-AMP-PNP (formed by maintaining an excess of AMP-PNP in the presence of S_1) with a binding constant of 1.1×10^5 M^{-1}. The binding constant of AMP-PNP to the binary complex of actin-S_1 was found to be 5.4×10^4 M^{-1}. From these binding constants, it can be calculated that, at a substrate concentration of 1.5 mM, all of the actin-S_1 will be in a ternary complex, which is in good agreement with *in vivo* data. Furthermore, from the two measured binding constants it is possible to calculate the binding constant for the association of actin with S_1 about which there is much debate. The calculated value of about 1×10^7 M^{-1} at 0.1 M KCl supports some previous values and disagrees with others. The data show further that the binary complex of S_1-AMP-PNP binds to F-actin in a 1:1 ratio with actin monomer. Studies underway with HMM-AMP-PNP seem to show almost the same binding constant as for S_1-AMP-PNP which suggests that only one of the two heads of HMM may bind to actin.

These, and other data, have all been integrated into a new model for the enzymatic cycle of actomyosin ATPase which correlates the intermediate states of the actin and myosin molecules with specific physical conformations of the actin and myosin in the thin and thick filaments of muscle *in vivo*. Much of the information gained from pre-steady state and steady state kinetic analysis can be directly translated into an understanding of the physiological process of muscle contraction.

Muscle contraction involves the participation of proteins other than actin and myosin, in particular tropomyosin and troponins. These proteins interact with actin in skeletal muscle and modify its interaction with myosin so as to confer a Ca^{2+}-requirement on the contractile process. The details of the interactions of these proteins are mostly unknown. Previous work in this laboratory demonstrated that muscle tropomyosin binds more weakly to *Acanthamoeba* actin (see section on Cell Motility) than to muscle actin but that, under conditions where both actins bound tropomyosin, tropomyosin inactivated the muscle acto-HMM ATPase activity and increased the *Acanthamoeba* acto-HMM ATPase activity. Extension of these studies, has now demonstrated that a slightly but significantly higher concentration of Mg^{2+} is required for

optimal binding of tropomyosin to Acanthamoeba actin than to muscle actin and that the binding in both cases is highly cooperative. It is possible to make random co-polymers for which the optimal Mg^{2+} concentrations lie between those for the homopolymers and whose abilities to activate the ATPase of HMM also suggest that each of the actins retains its unique properties in the copolymer despite the high degree of cooperativity between the muscle and Acanthamoeba actin monomers in the copolymer. Studies such as these, when correlated with the structure of the different actins, should be of great help in understanding the actin-actin interactions in the actin filament and the actin-tropomyosin interactions.

Cell Motility: Work in this and other laboratories for the past several years has established that all eukaryotic cells contain actin and myosin and that these proteins are very similar to, but different from, the corresponding proteins in skeletal muscle, the most highly evolved motility system. The non-muscle systems contain a number of other proteins, some of which, but not all, have muscle counterparts. The biochemistry and physiology of the numerous non-muscle motility systems are only very poorly understood. It is generally believed that, in contrast to muscle, much of the actin in non-muscle cells may be in a non-polymerized form. This would allow the cell to regulate the regions where motile processes occur by regulating the conversion of G-actin to F-actin, the functional form. Non-polymerized actin could occur either (1) because its concentration is below the critical concentration required for polymerization or (2) other factors keep the actin non-polymerized at concentrations where it would otherwise polymerize. Evidence has been obtained by some workers that spleen and other tissues contain a protein that forms a non-polymerizable complex with actin while others have claimed that embryonic chick brain and human platelets contain forms of actin that are incapable of normal polymerization even at very high concentrations.

We have now isolated actin from embryonic chick brain, human platelets, rat liver, and Acanthamoeba castellanii, by procedures developed in this laboratory, and demonstrated clearly that all polymerize in a manner qualitatively similar to muscle actin; first a nucleation step and then an elongation step. Quantitatively, the non-muscle actins are much more similar to each other than any is to muscle actin. However, all of the pure actins polymerize at concentrations much lower than the concentrations at which they exist in cell extracts (and presumably in the cell). These results leave little doubt that the polymerization of non-muscle actins is regulated by their interaction with other proteins in the cell. All the non-muscle actins activate muscle HMM ATPase more poorly than does muscle actin because of a lower K_{app} but the vertebrate non-muscle actins are more like muscle actin than like Acanthamoeba actin in this property. We have confirmed that the vertebrate non-muscle actins are mixtures of two species (β, γ) with isoelectric points more alkaline than the single species (α) in skeletal muscle actin and have found that Acanthamoeba actin is yet another species (δ) with an isoelectric point even more alkaline than those of γ-actin.

Several years ago we discovered that Acanthamoeba contains an unusual myosin that is much smaller than other myosins that have been described (180,000 daltons versus 450,000 daltons) and is a single-headed enzyme rather than a two-headed enzyme. Its heavy chain is about 140,000 daltons and it has two

light chains of about 14,000 and 16,000 daltons. We have now discovered the presence of a second myosin in the amoeba and have purified it to homogeneity. This Acanthamoeba myosin II, in contrast to Acanthamoeba myosin I, is a two-headed enzyme of native molecular weight about 350,000 and consists of two heavy chains of 170,000 and two 17,000-dalton light chains and two 17,500-dalton light chains. The two myosins differ in relative activities as Ca^{2+}-ATPases and K^{+}, EDTA-ATPase, substrate specificity and other properties. Only the previously described Acanthamoeba myosin I is activated by actin in the presence of the required cofactor proteins. We can find no evidence that one of these myosins is derived from the other or that both are derived from a still larger myosin with a molecular weight closer to that described for myosins from other sources. This is the first report of two myosins in a single cell and it suggests the obvious possibility that there may be different motile systems in the same cell for the different motile processes such as cell movement, cell division, chromosome segregation, phagocytosis, etc.

We have previously described the presence in Acanthamoeba of four proteins each of which causes the formation of a gel when added to F-actin. Acanthamoeba myosin II, but probably not Acanthamoeba myosin I, interacts with this gel to cause it to shrink (contract). In Acanthamoeba, we now know that the regulation of the polymerization state of actin will be controlled by unknown factors that interact with actin to keep it non-polymerized under conditions where the pure protein would polymerize and by at least four identified gelation factors that interact with F-actin. There are two myosins in the cell with which the F-actin will interact. In one case, Acanthamoeba myosin I, this actomyosin complex has much more Mg^{2+}-ATPase than the myosin alone if, and only if, protein cofactors are present. We are now purifying these cofactors. In the other case, Acanthamoeba myosin II, the actomyosin complex is only 1.5 to 2 times more active than the myosin alone and there may be unknown proteins that will stimulate the ATPase of that system.

Microtubule Polymerization: Cytoplasmic microtubules are major structural and functional elements in all eukaryotic cells. The microtubules are primarily polymeric forms of the dimer of α and β tubulin together with a number of accessory proteins that are essential to microtubule function. Microtubules rapidly polymerize and depolymerize and, as with the polymerization of actin filaments discussed above, the control of the organizational state of the tubulin dimer is essential to its physiological function. We understand very little of how the cell regulates the polymerization state of microtubules. One possibility is that the action of an enzyme might be involved, a specific tyrosine ligase that adds tyrosine to the C-terminal glutamate residue of α-tubulin in a reaction that requires only tyrosine, tubulin, ATP, and Mg^{2+}.

Tyrosine ligase has now been purified by scientists in this laboratory about 300-fold from calf brain but the enzyme is estimated still to be only 5% pure. The enzyme exists as a 1:1 complex with tubulin dimer for a complex molecular weight of 150,000. Tubulin dimer is 110,000 and the enzyme polypeptide is 35,000. The complex can be dissociated by high ionic strength, chromatography on DEAE-cellulose or organic solvents but it is unstable when freed of tubulin dimer. It is still not known if the complex is a simple enzyme-substrate complex or if the tubulin is an allosteric modifier of the enzyme.

The tyrosyl ligase reaction is reversible using the purified enzyme, as it is in cruder preparations (contrary to the claims of others), but crude extracts of brain do contain a second enzyme that is a specific detyrosylating carboxypeptidase that will also remove the tyrosyl group from the tyrosylated α-chain of the tubulin dimer. Whereas, reversal of the tyrosine ligase is optimal in 3 mM ADP, 10 mM Pi and 5 mM Mg^{2+}, the detyrosylating carboxypeptidase is stimulated by much higher Mg^{2+} concentrations and by GTP.

It has not been possible to tyrosylate more than 10 to 56% of the α-chains of tubulin. It is not known if the non-acceptor α-chains are inactive because the C-terminal glutamate is already blocked by something other than tyrosine (it cannot be tyrosine itself because tyrosine cannot be recovered by reversal of the tyrosine ligase reaction or by the specific carboxypeptidase) or if there are α-chains that do not have a C-terminal glutamate and are, therefore, not available as substrate.

The role of tyrosylation in the cell is still not known. Experiments in vitro have not shown any demonstrable effect on polymerization capability. One possibility is that the tyrosylation state is different for membrane-bound tubulin and cytoplasmic microtubule tubulin or in different functional states of tubulin that are still not known. Some indication has been obtained that the membrane-bound tubulin is a better acceptor of tyrosine groups than cytoplasmic tubulin, perhaps reflecting a difference in terminal tyrosine content.

It is also possible that there may be differences in tyrosyl ligase activity in different cells. Preliminary studies (with Dr. Marshall Nirenberg) suggest that the relative activities for several cultured cell lines are different; relative activities are: neuroblastoma cells 0.12, undifferentiated neuroblastoma-glial hybrid 0.05, differentiated hybrid 0.06, SV-40 transformed glial cells 0.13.

Electron Transport: Many of the components of the electron transport chain are cytochromes each of which contains a heme group, and has a characteristic absorption spectrum. Furthermore, each of the cytochromes has a different spectrum in the reduced and oxidized form. Therefore, it is possible theoretically to identify each of the cytochrome components of an electron transport system and to place them in proper sequence by kinetic analysis of the spectral changes during oxidation and reduction. If one starts with the oxidized chain, for example, the first spectral changes detected should be those resulting from reduction of the first cytochrome in the chain; the nature of the spectrum before and after reduction should identify the cytochrome. Previously, redox titrations were performed by mechanical titration with chemical reductants or oxidants. With such systems it is very difficult to maintain a fixed potential long enough to get a reliable spectrum and it is difficult to titrate the potential accurately.

A system has now been developed for microcomputer-controlled regulation of the redox potential of solutions through generation of an electrical current. This system is coupled to a spectrophotometer which then allows the experimenter to record the differences in absorption at a fixed wavelength for multiple repeated titrations of an oxidation-reduction system or, alternatively, to obtain and store a series of spectra at different fixed voltages. The computer-controlled system has been proven to work with four test substances of

increasing complexity (1) inorganic ferricyanide, (2) soluble cytochrome c, (3) insoluble cytochrome b_1, and (4) a suspension of fragmented E. coli membranes.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00401-11 LCB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Potentiometric studies of respiratory components of E. coli and rat liver mitochondria

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

PI: Richard W. Hendler, Head, Sec. on Membrane Enzymology LCB NHLBI

Other: A. Instrumentation
Tom Clem, Electrical and Electronic Engineering Section, Biomedical Engineering and Instrumentation Branch, Division of Research Services

B. Microcomputer Adaptation
David Songco, Computer Systems and Laboratory, Division of Computer Research and Technology

COOPERATING UNITS (if any)

None

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Laboratory of Cell Biology

SECTION
Section on Membrane Enzymology

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20014

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0.8	0.8	

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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 microcomputer-controlled system for controlling solution redox potentials by electric currents is described. This system, used with a spectrophotometer, studies the redox properties of substances that absorb light. The system performs repeated potentiometric titrations on a given sample or takes and stores a series of optical spectra at different voltages.

Project Description:

Objectives: In our previous report we defined various parameters for the optimal use of electric currents to influence the redox potential of aqueous solutions. Hardware interfaces to link the actual potentiometric experiment to a microcomputer were constructed and microcomputer programs to control two different kinds of experiment were written. The present report describes the integrated system and presents the first results obtained with this system.

General Description of the System: The preparation to be analyzed is placed in an optical cuvette which is then covered with a molded cap containing the following components:

1. A measuring platinum electrode paired with a reference Ag/AgCl electrode.

2. An oscillating, mixing, working electrode alternately paired with a working Ag/AgCl electrode and the reference electrode.

3. Gas inlet port.

4. Gas efflux and injection port.

The measuring electrodes are connected to a digital voltmeter. The optical cuvette is placed in a spectrophotometer for absorbance measurements. The overall system produces 4 analogue signals. 1) The redox potential of the solution. 2) The potential on the surface of the working platinum electrode. 3) The absorbance of the solution. 4) The wavelength for the absorbance. These analogue signals are interfaced to 4 channels of A/D (analogue to digital) conversion for their input to the microcomputer (MC) as digital numbers. The MC controls the magnitude of current passing through the solution by sending a number to a D/A converter which then controls a current generator connected to the working electrodes. The MC also sends out signals to do the following:

1. Move the recorder pen along the X-axis to record solution potential.

2. Raise and drop the recorder pen.

3. Start, stop, and control the direction of the wavelength scan drive motor.

4. Start and stop the mixing motor at a certain point in its oscillation. This is accomplished with a reflecting mirror on the bottom of a drive disc and a light-emitting and collecting diode. When the MC activates the circuit, the light beam is reflected onto a collector to control a relay which then stops the motor.

Kinds of Experiments Performed: Two kinds of experiment are performed. The first is a, or a series of, potentiometric titrations. The operator is prompted by the teletypewriter (TTY) to specify the following:

1. Low voltage limit.
2. High voltage limit.
3. Desired mv/min titration rate.
4. Pulse time duration.
5. Depolarization-equilibration time duration.
6. Number of successive titrations.
7. Amplification factors for each A/D conversion.

The ability of the system to carry out a depolarization and equilibration (item 5) is the key to the success of the entire approach. After each pulse of current, the working Pt electrode retains a large residual charge which must be neutralized before the system components equilibrate. The MC notes the magnitude of this charge by comparing both the measuring and working Pt electrodes relative to the reference electrode. The magnitude of the polarization charge is used in appropriate equations to generate small spurts of neutralizing currents which dissipate the charge. The MC carries out the specified titrations alternating between reductive and oxidative cycles. The TTY prints in tabular form a record of the times of data collection, the sequential number of the data points, the magnitude of the pulse current, the rate of change of solution potential, the difference from the desired rate, the working electrode voltage, the measuring electrode voltage, and the optical absorbance of the solution. A complete record of the data is stored in the MC and transmitted to the Decsystem 10 computer for further computation.

The second type of experiment collects a series of spectral scans at programmed voltage intervals. The operator is prompted by the TTY to specify the following:

1. Starting voltage.
2. Voltage increment.
3. Voltage limit.
4. Starting wavelength.
5. Wavelength increment for the collection of optical density data.
6. Upper wavelength limit of the scan.

The MC attains the desired voltages, depolarizes and equilibrates, takes and stores spectral scans, and informs the operator of any voltage changes during the scan. At the end of the experiment, the data is transferred to the Decsystem-10 computer for further processing. The spectra (i.e. wavelength vs absorbance) are plotted against the solution potential in a 3-dimensional plot to produce a surface which shows the response of every light-absorbing component to changes in oxidizing potential (i.e. to reveal their electron affinities).

Results obtained with the system: The system was tested with a soluble inorganic substance (ferricyanide), a soluble protein (cytochrome c), and an insoluble respiratory pigment (cytochrome b_1) in a suspension of E. coli membrane fragments. The need for and influence of soluble redox mediators were also studied. Ferricyanide was cycled through 5 titrations alternating between oxidations and reductions. There was excellent agreement for all 5 titrations and between the actual data and the theoretically expected data according to the Nernst equation. Mediators had no effect on the fidelity of the data obtained but they played a very important role in stabilizing solution potentials. With cytochrome c, good agreement was obtained in pairs of oxidative and reductive titrations and the Em values obtained agreed with literature values. Mediators, in addition to stabilizing the voltage, helped to maintain the agreement between oxidative and reductive titrations. With the membrane-bound cytochrome b_1, the system revealed 3 potentiometric species in agreement with our previous results obtained with manual chemical titrations. The two higher Em species were the same as previously found. The low Em species was somewhat lower in the current studies. Redox mediators were absolutely essential to obtain reproducible data.

In the experiment of the second kind, a surface topography for $K_3Fe(CN)_6$ was obtained and the rise of its peak was found to obey the Nernst relationship. With E. coli membranes, the α absorbance of cytochromes b_1, a_1, and d stood out as structural features in a spectra versus voltage surface. Cytochrome b_1 was shown to obey the Nernst relationship. Previous work with manual titrations of cytochrome d revealed an atypical response of this cytochrome to voltage change. The surface map generated in the present work showed that a hitherto unrecognized component, obeying the Nernst law, rises up at the wavelength used to mark the "valley" for cytochrome d. Therefore, as this feature becomes more prominant, the peak to valley height of cytochrome d appears to diminish with decreasing voltage.

Proposed Course of Project: In principle, the system we have developed could be used to take any respiratory chain and allow one to identify every component which absorbs light and changes its state of oxidation according to Nernst's law. This means all components, known and unknown making up the chain. If these components change their absorbance or redox potential as a state of energization of the system, this should also be seen. One complication to the development of this approach is the fact that the redox mediators contribute significantly to the spectrum in the region that accounts for flavoproteins, nonheme iron proteins and Soret absorptions of the cytochromes. In collaboration with a mathematician, Richard I. Shrager, we will try to develop an

approach which will allow us to take into account all of the mediators and then resolve the respiratory chain into all of its components. If we are successful we shall proceed to fully analyze E. coli and mammalian mitochondrial systems.

Publications:

1. Hendler, R. W., Songco, D. and Clem, T.: Automated electrodic potentiometry system. Anal. Chem., accepted for publication.

2. Hendler, R. W.: Automated electrodic potentiometry of potassium ferricyanide and respiratory components. Anal. Chem., accepted for publication.

Other:

An employee invention report has been filed with Mr. Norman J. Latker, patent counsel of HEW.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00402-05 LCB

PERIOD COVERED
July 1, 1976 to September 30, 1977

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, Sec. on Membrane Enzymology LCB NHLBI

Raymond Scharff, Chemist LCB NHLBI

Musetta Hanson, Staff Fellow LCB NHLBI

COOPERATING UNITS (if any)
None

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.9	1.9	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) DNA polymerase I and exonuclease V can be combined in the presence of DNA followed by DNase to form a high molecular weight form of DNA polymerase which uses native DNA in an ATP-stimulated DNA-polymerizing system. The synthetic complex may be related to a natural complex found in E. coli. The natural complex exists bound to DNA which is in turn bound to a sedimentable structure from which it can be released by incubating at 30°C. Natural complex activity is present in a rec B_{21} C_{22} mutant which lacks exonuclease V activity. The DNA-stimulated ATPase activity of the rec BC enzyme may be essential for complex activity.

PHS-6040
(Rev. 10-76)

Project Description:

Background: *E. coli* contains a high molecular weight form of DNA polymerase I which acts with a native DNA template in an ATP-stimulated DNA polymerizing system. Previous studies indicate that the polymerase is complexed with exonuclease V (*rec* BC enzyme). The current report describes the attempted synthesis of complex from the component enzymes and further studies with the biological complex.

Major Findings:

Attempts to form complex from DNA polymerase I and exonuclease V: The criterion for presumptive complex formation was the *de novo* appearance of ATP-stimulated polymerase activity, using native DNA as template, in an elution volume from a column of Biogel A 1.5 m that corresponds to a relatively high molecular weight (i.e., several hundred thousand instead of the 109,000 characteristic of free polymerase I). We have been successful in meeting this criterion and the presumptive complex can be re-chromatographed on another column, eliminating the explanation that it represents a fortuitous mixture of overlapping separate peaks. The formation of such synthetic complex requires the participation of DNA and DNase. Testing various orders of addition and pre-incubations we have found that the best sequence is to expose the polymerase to DNA, followed by incubation with DNase, and finally the addition of exonuclease V. Exposing both polymerase and exonuclease V to DNA followed by DNase lowered the yield by about 25%. Treating DNA with DNase prior to adding the component enzymes reduced the yield by about 75% which was the same result obtained by using polymerase and DNase alone. The finding of some apparent complex activity in a sample of commercially purified polymerase is consistent with the idea that complex represents a real biological entity that is not so easily removed from DNA polymerase itself. The relation, if any, of the synthetic and natural complexes has not yet been established.

Further studies with biological complex: In the isolation of complex from a cell homogenate, a 20,000 x g supernate is passed over a Biogel A 50 m column. The complex emerges in the void volume. Non-denaturing polyacrylamide gels, electrophoresed in pairs (with and without DNase treatment), show that essentially all of the Coomassie blue-positive bands enter the gels without prior DNase treatment and that these bands elute in the included volume of the Biogel A 50 m column, which does not contain complex. The void volume which contains all of the complex has a very low content of Coomassie blue-positive material. The supernatant fraction obtained by incubating the void fraction at 30° and centrifuging at 105,000 x g has essentially all of the complex activity and only one or two major Coomassie blue-positive bands compared to more than 20 in the material applied to the A50 m column. These bands cannot be complex because, until DNase is used, the complex is still associated with DNA. After DNase treatment of the incubated supernate, a new band appears in the acrylamide gels. An *in situ* polymerase assay will be required to see if this band could be complex.

The complex present in the A 50 m void fractions is sedimented in 60 min at 105,000 x g. The incubation of the void fractions for 90 min at 30° (but not at 5°C) causes the liberation of complex from the material which sediments in the ultracentrifuge, but leaves it in a form still excluded from a Biogel A 1.5 m column (membranes ?). There is a similar form of complex present in the cell homogenate which represents about 20% of the total, does not sediment at 105,000 g and is excluded from Biogel A 1.5 ml and A 50 m columns. These results suggest that the complex exists in the cell in at least two stages of structured relationships. 1) Associated with free DNA, and 2) associated via DNA to a large sedimentable structure. The sedimentable form is converted to the non-sedimentable form by incubation at 30°. In previous work, the free complex was liberated both from DNA and sedimentable structures by DNase treatment. If the complex is first liberated from the sedimentable form by a preliminary incubation and then treated with DNase, the recovery of free complex (i.e., included volume from Biogel A 1.5 m) is doubled.

A potential cause for concern in this work has been the possibility that exonuclease V can simply activate native DNA by nicking and gapping so that, like pancreatic DNase, it makes DNA a better template for DNA polymerase. We have used the double mutant rec B_{21} C_{22} and established that the homogenates do not contain any detectable ATP-stimulated nuclease (exonuclease V) activity. Nonetheless, we have demonstrated that complex is present and can be isolated. The most likely and most interesting explanation is that the DNA-stimulated ATPase activity of the rec BC enzyme is still present and that it is this activity which is required for the complex activity. There is a high background of DNA-independent ATPase activity, but there also is a small peak of DNA-dependent ATPase activity which overlaps complex activity in the fractions eluted from Biogel A 1.5 m both from the rec BC mutant and the wild type. Additional studies are required before we can say that the two activities are related.

Proposed Course of Project: The purification of natural complex to homogeneity will be pursued. Pure natural complex will be compared with complex synthesized from exonuclease V and polymerase I. We will try to establish whether or not the activity of the complex depends on the presence of the DNA-stimulated ATPase activity of the rec BC enzyme.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00403-03 LCB

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Differential Scanning Calorimetry of Fibrinogen

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

PI: Elemer Mihalyi, Staff Investigator LCB NHLBI

Other: None

COOPERATING UNITS (if any)

Dr. John W. Donovan, Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Berkeley, California 94710

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Laboratory of Cell Biology

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	None

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)

Differential scanning calorimetry detected an increase of the heat of unfolding of the D-domain of fibrinogen during clotting. This is much slower than the primary clot formation, but is dependent on thrombin concentration and requires the presence of calcium.

Project Description:

Objectives: In our previous study (Donovan, J.W. and Mihalyi, E.: PNAS, 71: 4125, 1974) it was found that upon clotting of fibrinogen, and much more slowly than in the clotting reaction, an increase in the heat of unfolding of the D-domain of the molecule occurs, connected with a shift of the transition temperature of about 10°. The nature of these changes has been investigated in the present studies.

Methods Employed: Differential scanning calorimetry.

Major Findings: The above mentioned changes were observed with Sigma thrombin of approximately 10% purity. The experiments were repeated with a very pure human thrombin of better than 95% purity obtained from Dr. Fenton (N.Y. State Department of Health, Albany, N.Y.) and the same results were obtained. In further experiments, it was found that the effect is absent in the presence of EDTA, but it can be restored by calcium. Its magnitude is dependent on the calcium concentration and maximal effect is obtained at 10^{-3} M concentration. Calcium had no effect at all on the differential calorimetric scans of fibrinogen. The increase of enthalpy of unfolding and of the transition temperature was a first order process and its rate depended on the thrombin concentration. Thus, this effect appears to follow some thrombin mediated change in the fibrinogen molecule, but this in itself is not sufficient for the structural change. The latter requires also the presence of calcium ions.

Significance to Biomedical Research and Institute Program: Elucidation of the mechanism of "in vitro" clotting of fibrinogen may help understand thrombus formation "in vivo".

Proposed Course of Project: The conformational changes of clotted fibrinogen will be related to calcium binding of the protein. Studies will be performed to identify the nature of the thrombin action, which is much slower, and seems to be not related to the splitting off of fibrinopeptides by this enzyme.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00404-18 LCB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Proteolytic Fragmentation of Fibrinogen

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

PI: Elemer Mihalyi, Staff Investigator LCB NHLBI

Other: Masatsugu Kisaragi, Visiting Scientist LCB NHLBI

David Towne, Chemist LCB NHLBI

COOPERATING UNITS (if any)
None

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National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20014

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2.3	2.3	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) A method was worked out for obtaining controlled serial digests of fibrinogen using the pH-shift of buffered solutions to monitor the progress of digestion. This was applied to comparative investigation of the digestion of normal and abnormal fibrinogens. The effect of sodium dodecyl sulfate (SDS) on the esterase activity of trypsin was investigated. This was necessary for the evaluation of the effectiveness of SDS in inhibiting the enzyme in gel electrophoresis of serial protein digests. SDS has an immediate and a progressive inactivating effect. These are reversed to various extent at high substrate concentrations. The inhibition is not sufficient to assure complete arrest of the digestion in SDS-gel electrophoresis experiments.

Project Description:

Objectives: 1) Detailed kinetic studies of the proteolytic fragmentation of fibrinogen have been used for structural characterization of the molecule. These studies could be useful also for study of the abnormal fibrinogens. However, the techniques for obtaining the serial digests would be difficult to apply to these because of the limited supply of material. Therefore, in the present studies techniques were worked out for obtaining precise results with small amounts of material.

2) Sodium dodecyl sulfate (SDS) is a powerful protein denaturing agent, nevertheless, proteolytic activity was reported in its presence. Most of these studies employed polyacrylamide gel electrophoresis to demonstrate proteolysis. In the present studies more quantitative data were sought by investigating the effect of SDS on the esterase activity of trypsin. Activity was determined by the pH-stat method.

Methods Employed: Proteolytic digestion monitored by either the pH-stat, or by the pH-shift method; sodium dodecyl sulfate-polyacrylamide gel electrophoresis with UV-scanning; computer resolution of the kinetic data.

Major Findings: 1) pH-stating by scaling down the size of the reaction vessel and syringe could be used to provide accurate results with 1 ml of solution of 10 mg per ml, fibrinogen. However, successive removal of samples disturbed the system to such extent that mathematical correction for the stepwise decrease of the amount of substrate was not satisfactory. Instead of this the pH-shift method was employed. Adding sufficient amount of buffer (pH 8 Tris in this case) reduces the pH change during digestion to the desired extent (usually 0.1 pH unit). Within this range and at a pH close to the pK of the buffer, the pH change and the amount of H^+ produced are in linear relationship. This was proved by the correspondence of pH-shift and pH-stat curves. A correction was necessary for the former, because the buffering capacity of the system increases with the number of bonds cleaved. Correction, however, is minimal. With the pH-shift method samples can be removed with no disturbance to the recording of the digestion, since obviously the pH of the system is independent of the volume of the sample.

With this method the digestion of normal human fibrinogen by plasmin was compared with the digestion of the abnormal fibrinogen "Chapel Hill". Differences were found in the rate of fragmentation of the molecule.

2) Esterase activity of native trypsin on tosyl arginine methyl ester (TAME) was activated slightly (about 8%) at low concentrations of SDS. At higher concentrations of SDS activity was inhibited, but the extent of inhibition depended on the concentration of substrate. At 1% SDS (0.035 M) and 10^{-3} M or 10^{-2} M TAME concentration substantial activity was observed, whereas at 0.1 M SDS and 10^{-4} M TAME concentration the enzyme was completely inhibited.

When trypsin was incubated with SDS at pH 3 for 5 min before it was added to the assay mixture a recovery of the rate of hydrolysis was observed:

the initial rate was slow, but the rate increased gradually with time. The recovery was observed even after an overnight incubation at room temperature under the above conditions. In these experiments the assay mixture contained no SDS. When the latter was included in the mixture recovery was not observed at moderate TAME concentrations, but was present when 10^{-2} M TAME and 0.1% SDS were used. Apparently the substrate exerts a refolding or protecting effect opposed to the unfolding by SDS.

Incubation at neutral pH causes irreversible loss of activity by autodigestion of trypsin. This was observed also at 0.1% SDS concentration at pH 7. At higher concentrations, SDS appeared to prevent autodigestion, decrease of activity being slower under these conditions.

Significance to Biomedical Research: Study of abnormal fibrinogens may help in understanding the coagulation defects associated with these.

Proposed Course of Project: These studies will be extended to other abnormal fibrinogens with the aim of determining whether or not the genetic structural modification affects the regions of the molecule that are involved in the fragmentation reaction.

Studies on normal fibrinogen and its proteolytic fragments will be continued using classical hydrodynamic methods. These studies are necessary for the construction of a model which reconciles all the conflicting data and theories of this molecule.

Publications:

1. Mihalyi, E., Weinberg, R. M., Towne, D. W. and Friedman, M. E.: Proteolytic fragmentation of fibrinogen. I. Comparison of the fragmentation of human and bovine fibrinogen by trypsin or plasmin. Biochemistry 15: 5372-5381, 1976.

2. Shrager, R. I., Mihalyi, E. and Towne, D. W.: Proteolytic fragmentation of fibrinogen. II. Kinetic modeling of the digestion of human and bovine fibrinogen by plasmin or trypsin. Biochemistry 15: 5382-5386, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00405-03 LCB

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Circular Dichroic Studies on Denatured Proteins

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

Frederick H. White, Jr. Staff Investigator LCB NHLBI

COOPERATING UNITS (if any)

None

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Laboratory of Cell Biology

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Section on Cellular Biochemistry and Ultrastructure

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20014

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1	1	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

It has long been established that development of conformational structure is dependent on amino acid sequence, although the exact relationship (which would make possible the precise prediction of native conformation from primary structure) remains unclear. It has long been believed that native conformation develops from the randomly coiled chain of the denatured (or newly synthesized) protein, by a process of "nucleation" which would involve a progressive folding, governed by thermodynamic factors, until the native state is reached. This hypothesis rests on the assumption that the denatured protein exists predominantly as a random coil. However it has recently been observed in this and other laboratories that lysozyme, denatured by reduction, contains secondary structure in amounts easily detectable by circular dichroism. This observation has now been extended to other proteins, which are: chymotrypsin, cytochrome c, papain, elastase, staphylococcal nuclease, and ribonuclease. In general their 'denatured' structures are characterized by lowered α-helix and either constant or increasing β structure. Thus, there appears to be a more complex folding mechanism than would be suggested by a process of nucleation. The sequence of events from the 'denatured' structure to the native conformation will be examined to shed more light on the folding mechanism.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To examine the conformational structures of several denatured proteins as they relate to the folding process.

Methods Employed:

1. The procedures for reduction of disulfide bonds and for reaction of the resulting SH groups with iodoacetate or iodoacetamide are similar to those already published (F.H. White, Methods in Enzymology 25: 387, 1972).

2. A Beckman automatic amino acid analyzer was used for verification of complete reaction with the alkylating agent (J.V. Benson and J.A. Patterson, Anal. Biochem. 37: 1108, 1965). The level of reduction was determined by titration with 5,5'-dithio-bis-(2-nitrobenzoic acid) (Ellman, Biochem. & Pharmacol. 1: 88, 1961).

3. The Cary Recording Spectropolarimeter with a Model 6001 circular dichroic attachment was used for all studies of circular dichroic behavior.

4. The development of basis spectra and processing of data from CD studies were accomplished with the PDP-10 Computer, employing the MLAB curve fitting system.

5. Gel filtration was carried out on native and denatured proteins with Sephadex resins (G-25 and G-100) by established procedures.

Major Findings:

1. A computer procedure has been elaborated for derivation of basis spectra used in quantitation of CD data.

2. With the above basis spectra, a further study (continued from previous Annual Report (Z01 HL 00405-02 LCB) has been completed on six proteins in the native and denatured states. These are chymotrypsin, cytochrome c, papain, elastase, staphylococcal nuclease, and ribonuclease. All denatured proteins exhibited measurable amounts of secondary structure. The results of quantitation are in many cases quantitatively different from earlier findings carried out with published basis spectra but nevertheless confirm the observation that denaturation involves a loss of α-helix (for some proteins virtually complete) while β structure remains constant or increases.

3. Further characterization of the 'denatured' structure has been carried out on reduced carboxamidomethyl lysozyme, reduced carboxymethyl lysozyme, reduced carboxyamidomethyl ribonuclease, and acid-denatured nuclease.

a. Nearly complete removal of the 'denatured' structure can be achieved by further randomization with guanidine or urea, by heating, and by acidification.

b. The 'denatured' structure is completely reversible from urea and guanidine solution, from heating as high as 60°, and from acidification as low as pH 1.2.

c. The 'denatured' structure is monomeric with respect to gel filtration (single peak emerges near the native peak) and with respect to CD behavior over a 15-fold range of concentration.

d. Conclusions: The 'denatured' conformational structure does not appear to result from polymerization. It is the preferred structure of the reduced state, rather than merely a residual structure, left over from the breakdown of native conformation.

4. Most of the denatured forms of this study were insoluble above pH 3.5 and were thus studied at or below this level. Most of these, however, are renaturable only on the alkaline side (approximately pH 8), and since the objective is to examine how such structure relates to the folding process, a means was sought to solubilize the denatured proteins at pH 8. It was found that, with careful manipulation, it was possible to solubilize the carboxymethyl derivative of reduced lysozyme at pH 8. CD study showed that there is little variation in the 'denatured' structure of this derivative with pH. Thus the structure is present at the start of the folding process that produces the native protein.

Significance to Biomedical Research and Institute Program: The point of view has long been established that reduction of disulfide bonds would result in the formation of a random coil (e.g., see C. Tanford, Adv. Prot. Chem. 23: 122, 1968). The "nucleation hypothesis" summarized by (C. B. Anfinsen, Science 181: 223, 1973), in fact, evolved during a period when this principle was widely accepted and thus assumes a process of progressive folding, as dictated by the primary structure and thermodynamic factors, from the randomly coiled state. The results found here suggest a modification of the hypothesis, as a consequence of observations that secondary structure (in preference to random coil) exists prior to the folding process that produces the native conformation. Thus the discovery of 'denatured' structure may be regarded as a step leading to a better understanding of the process by which native structure is gained within the newly synthesized protein chain in the living cell.

While it is reasonable to assume that a process of nucleation accounts for development of the 'denatured' structure from the random coil, it is now problematic how the native structure develops from the 'denatured' structure. The necessity for gaining α-helix indicates that it involves more than a folding of widely separated domains. The necessity for losing β structure indicates that a continued progressive folding, as would be governed by a process of nucleation, does not suffice to explain the formation of native structure.

Proposed Course of Project: 1. Attempts will be made to examine the sequence of events in the folding process between 'denatured' structure and native

structure. This investigation will involve isolation of intermediates at various stages of oxidation and correlating with secondary structure.

2. Results of CD studies will be compared with those of other methods (e.g., raman spectroscopy).

3. The question of where the increased β structure occurs within the denatured molecule will be examined and attempts made to correlate its presence with various methods that have been used for predicting conformational structure from amino acid sequence. Various degradative procedures may be used for this purpose.

Publications:

White, F. H., Jr.: Studies on secondary structure in chicken egg-white lysozyme after reductive cleavage of disulfide bonds. Biochemistry 15: 2906-2912, 1976.

PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

Z01 HL 00409-07 LCB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

The 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, Head, Section on Cellular Physiology LC NHLBI

Other: (Stopped flow studies) Stephen P. Chock, Staff Fellow LC NHLBI

(Theoretical work) Terrell L. Hill LMB NIAMDD
(Stopped flow studies) P. Boon Chock LB NHLBI

COOPERATING UNITS (if any)

Laboratory of Molecular Biology, National Institute of Arthritis, Metabolism and Digestive Disease; Laboratory of Biochemistry, National Heart, Lung, and Blood Institute

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Section on Cellular Physiology

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.4	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)

Our major effort over the next year will still be to determine the relationship of the initial Pi burst to the transition from the refractory to the non-refractory state. At the present time using subfragment-one (S-1) at 15° in the absence of KCl, we find that the rate of the initial Pi burst is 2 to 3 times the rate of the transition from the refractory to the non-refractory state. This is not a large enough difference to be certain that they are separate steps. Therefore we plan to repeat these experiments using chymotryptic digestion to prepare S-1 which can then be separated into two fractions each of which contains a different alkalai light chain and shows a different actin-activated ATPase rate. By comparing the initial Pi burst and the actin activation at varying pH and ionic strength with these preparations we should be able to determine the relationship between the initial Pi burst and the transition from the refractory to the non-refractory state quite accurately. In our theoretical work with Terrell Hill, we plan to quantify our cross-bridge model to determine whether it will fit current physiologic data without making any further assumptions.

Project Description

Objectives: It is now generally recognized that contraction of muscle involves the interaction of the two proteins actin and myosin with ATP. It is therefore of considerable importance to determine the nature of the interaction which occurs between actin and myosin in vitro as ATP is hydrolyzed. In particular it is of importance to elucidate the steps occurring during the hydrolytic cycle, since these steps may correspond to the steps of the contractile cycle in vivo. This is difficult to accomplish with myosin because it occurs as insoluble filaments at low ionic strength. However, heavy meromyosin (HMM) and subfragment-1 (S-1), double and single headed proteolytic digestion products of myosin respectively, are soluble at low ionic strength and therefore their interaction with actin can be more easily studied. In the present study we continued our investigation of the refractory state of HMM and S-1, a state which we discovered occurs during the cycle of interaction of myosin with actin and ATP during which the myosin head is unable to bind to actin. Only when the refractory state transforms to the non-refractory state is the myosin able to interact with actin and the transition from the refractory state to the non-refractory state seems to be one of the major rate-limiting steps in the cyclic interaction of myosin with actin and ATP. The occurrence of a refractory state has considerable implications for the actin-myosin cycle which occurs in vivo and therefore in the present study we devoted considerable effort to proving its existence and determining its chemical nature using stopped-flow techniques. In addition, in collaboration with Terrell Hill, NIAMD, we have developed a complete model of cross-bridge actins which shows the significance of the refractory state for muscle contraction in vivo.

Methods Employed and Major Findings: We previously showed, using pre-steady state techniques, that, in a single cycle of ATP hydrolysis by acto-S-1 at 5°, the maximum rate of rebinding of the S-1 to actin is about 1 sec^{-1}, the same as the maximum actin activated ATPase rate (V_{max}) determined from steady-state measurements. In the present study we repeated these experiments at 15°. Our results show that, as at 5°, the rate of rebinding of S-1 to actin is equal to the steady-state ATPase rate, levelling off at high actin concentration at a rate of about 5 sec^{-1}, the same rate as V_{max} determined from steady state measurements. These results suggest that, at 15°, as at 5°, the rate-limiting step in the cycle of ATP hydrolysis occurs while the S-1 is detached from the actin, i.e., the rate-limiting step is what we have termed the transition from the refractory to the non-refractory state.

In an effort to determine the nature of the refractory and non-refractory state, we next compared, under the same conditions, the rate of binding of ATP to S-1, the rate of the subsequent hydrolysis step, and the rate of the accompanying fluorescence change. The following scheme has been proposed for the myosin ATPase by Bagshaw et al.:

$$M+T \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} M\cdot T \overset{k_2}{\rightarrow} M^*\cdot T \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} M^{**}\cdot D\cdot Pi \longrightarrow M+D+Pi$$

where M = myosin, T = ATP, D = ADP and the transition from M·T to M*·T represents an irreversible conformational change in the myosin molecule. One important question about this scheme is how much of the fluorescence change which accompanies ATP binding is due to the formation of M*·T and how much is due to the formation of M**·D·Pi. A second key question is how the rate of formation of M**·D·Pi relates to the rate of the transition from the refractory to the non-refractory state. In the present study we measured the rate of formation of M*·T by mixing S-1 with γ^{32}P-ATP followed by a cold ATP chase at varying times, using a 3-syringe stopped-flow apparatus. The rate of formation of M**·D·Pi was measured in the same apparatus using an acid quench, while the rate of the fluorescence change was measured in a 2-syringe stopped-flow apparatus. As expected at 15°, pH = 8, 0.5 M KCl, where the rate of the fluorescence change is directly proportional to the ATP concentration, the rates of formation of both M*·T and M**·D·Pi are essentially equal to the rate of the fluorescence change. However, at 15°, pH = 7, in the absence of KCl, where the rate of the fluorescence change at 80 μM ATP is almost independent of the ATP concentration, the rate of formation of M*·T (>100 sec^{-1}) is more than 4 times faster than the rate of the fluorescence change (25 sec^{-1}) which is approximately equal to the rate of formation of M**·D·Pi (20 sec^{-1}). Therefore, the transition from M*·T to M**·D·Pi may make a significant contribution to the fluorescence change observed when ATP binds to myosin. Furthermore, the maximum rate of the fluorescence change may represent the rate of formation of M**·D·Pi (k_3+k_{-3}) rather than the rate of formation of M*·T (k_2).

We also find that, under these conditions, the equilibrium constant between M*T and M**·D·Pi is about 1.5. Since the rate of formation of M**·D·Pi (k_3+k_{-3}) is about 20 sec^{-1}, k_3, itself, must be about 13 sec^{-1} which is only about 2.5 times faster than the rate of the transition from the refractory to the non-refractory state (5 sec^{-1}). It is therefore possible that, at very low ionic strength, the transition from M*T to M**·D·Pi at least partly represents the transition from the refractory to the non-refractory state. If this is indeed the case, it raises interesting questions about the rate-limiting step at higher ionic strength and higher pH, i.e., 0.1 M, pH 8, where the rate of the transition from M*T to M**·D·Pi is markedly increased.

In our theoretical work with Terrell Hill, we have now completed our paper describing a complete cross-bridge model of muscle contraction based on our biochemical scheme for the actomyosin, ATPase activity. This paper is now in press. In our model, the elasticity of the cross-bridge is provided by postulating that the myosin head on the actin has the ability to rotate smoothly about two most stable bond angles, e.g., 90° and 45°, one angle for each attached state. In either state when the myosin head rotates away from its optimal angle, it exerts force. Unlike the Huxley-Simmons model, there is no independent elastic element in this model. Therefore, the transition between the two attached states does not involve, and hence is not slowed down by, one dimensional Brownian motion. This allows the model to have only two attached states. Since in this model the myosin head smoothly rotates in the actin site, we postulate that the rate of transition between the various cross-bridge states change gradually as the orientation of the myosin head changes on the actin. Whether this, in fact, occurs will have to be tested in future biochemical

experiments where the orientation of the myosin head on the actin might be expected to change, perhaps using ATP analogues.

One other project in collaboration with Sally Mulhern which was completed this year was our study on whether various types of sulfhydryl reagents all have the same effect when they block the SH_1 group of myosin. Our results suggest that all of the sulfhydryl reagents have very similar effects. This work has been written up and will be submitted for publication shortly.

Significance to Biomedical Research and Institute Program: This work is aimed at gaining a better understanding of the basic mechanism of muscle motility and the control of this motility, phenomena which occur not only in skeletal muscle, but also in such diverse systems as cardiac muscle, arterial smooth muscle, platelets and perhaps within all cells where protoplasmic streaming occurs.

Proposed Course of Project: Our major effort over the next year will still be to determine the relationship of the initial Pi burst to the transition from the refractory to the non-refractory state. At the present time using subfragment-one (S-1) at 15° in the absence of KCl, we find that the rate of the initial Pi burst is 2 to 3 times the rate of the transition from the refractory to the non-refractory state. This is not a large enough difference to be certain that they are separate steps. Therefore we plan to repeat these experiments using chymotryptic digestion to prepare S-1 which can then be separated into two fractions each of which contains a different alkalai light chain and shows a different actin-activated ATPase rate. By comparing the initial Pi burst and the actin activation at varying pH and ionic strength with these preparations we should be able to determine the relationship between the initial Pi burst and the transition from the refractory to the non-refractory state quite accurately. In our theoretical work with Terrell Hill, we plan to quantify our cross-bridge model to determine whether it will fit current physiologic data without making any further assumptions.

Publications:

Eisenberg, E. and Hill, T. L.: A cross-bridge model of muscle contraction. Progress in Biophysics and Biophysical Chemistry, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00410-04 LCB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Mechanism of Myosin and Actomyosin-Mg-ATPase

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

PI: Stephen P. Chock, Staff Fellow LCB NHLBI

Evan Eisenberg, Head, Section on Cellular Physiology LCB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Section on Cellular Physiology

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.8	.8	None

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 most crucial point we will investigate is whether the fraction of subfragment-one (S-1) which neither binds ATP irreversibly nor shows the initial Pi burst is, in fact, denatured. It is of importance to make certain that S-1 preparations can be prepared which have very little of this fraction. Purification of the S-1 or a DEAE cellulose column as well as preparation of S-1 with chymotrypsin rather than papain, should help resolve this question. In addition, heavy meromyosin and myosin itself will be studied. It is also important to make certain that the fraction of S-1 which does not show the initial Pi burst can still bind ATP reversibly. If it does so, it will be of interest to study the ATPase properties of this fraction both with and without actin present. Even if it is denatured, its properties may aid us in understanding the role of initial Pi burst in undenatured myosin. Another aspect of this project which requires further study is our finding that H^+ release accompanies the hydrolysis of ATP in the initial Pi burst. It has been reported that the binding of ADP on the ATP analogue AmPPNP also causes release of H^+ and whether this is, in fact, the case will require further investigation.

120

Objectives: Recent progress in our understanding of the mechanism of muscle contraction has been significantly advanced by the use of steady state and presteady state kinetic studies of both the myosin and actomyosin Mg-ATPase in vitro. The use of the presteady state approach, especially, has led to the discovery of various kinetic intermediates through which the enzymes pass during each cycle of ATP hydrolysis. However, there are still many disagreements among laboratories about the nature of the intermediates and about the interpretation of various experimental observations. In regard to the myosin ATPase in the absence of actin, one of the major questions is whether the two heads of each myosin molecule are identical in their kinetic properties. Another important question is the nature of the conformational change which occurs when ATP binds to myosin. Fluorescence and absorbance changes occur as well as H^+ release but it is not clear whether these changes are due to the binding of ATP or to the subsequent rapid hydrolysis of ATP on the surface of the myosin molecule which has been called the initial Pi burst. In the present study we investigated these questions to gain a better understanding of the myosin kinetic cycle.

Methods Employed and Major Findings: In a lengthy series of paper, Y. Tonomura and his collaborators have suggested that the two myosin heads differ in their kinetic properties. They argue that one head binds ATP essentially irreversibly and then hydrolyzes it rapidly in an initial Pi burst while the second head binds ATP more weakly and does not hydrolyze it in an initial Pi burst. This model predicts that the molar ratio of ATP irreversibly bound and hydrolyzed in the initial Pi burst to total amount of isolated myosin heads or subfragment-1 (S-1) present should be about 0.5. In the present study we measured the magnitude of the irreversible ATP binding by mixing S-1 with γ-P^{32}-ATP followed by a cold ATP chase at varying times, using a 3-syringe stopped-flow apparatus. With this model, only the irreversibly bound γ-P^{32}-ATP is hydrolyzed - the reversibly bound γ-P^{32}-ATP will exchange with the cold ATP and be diluted so it is not hydrolyzed. The initial Pi-burst was also measured in the 3-syringe apparatus using an acid quench rather than a cold ATP quench.

Our results suggest that with most S-1 preparations, 80% of the S-1 binds ATP irreversibly, and about 70% of this irreversibly bound ATP is rapidly hydrolyzed in the initial Pi-burst. However, in some preparations only 50-60% of the ATP appears to be irreversibly bound with a proportional reduction in the magnitude of the initial Pi-burst. Nevertheless, fluorescence titration experiments suggest that almost all of the S-1 is binding ATP in these preparations. Therefore, presumably, some of this S-1 is binding ATP reversibly as Tonomura suggested. However, it definitely does not appear to be half of the S-1. Furthermore, it may well represent denatured S-1. We are presently investigating this question in more detail.

The second question we investigated in this project is the cause of the H^+ release and absorbance changes which occur when ATP binds to myosin. As we described in Project Report # Z01 HL 00409-07 LCB, our fluorescence stopped-flow studies in combination with our 3-syringe stopped-flow experiments suggested that a large fraction of the fluorescence change observed when ATP binds to myosin is not due to the binding of ATP itself, but to the next step-

the initial Pi burst. It therefore becomes of interest to determine if the H^+ release and absorbance changes which accompany ATP binding are also due to the initial Pi burst. We previously studies this H^+ release and found that 0.4 moles of H^+ were released per mole of ATP which bound to myosin.

Since we have previously shown that both the rate of the initial Pi burst and the rate of the fluorescence change level off as the ATP concentration is increased while the rate of ATP binding itself increases rapidly, we tested whether the rate of the H^+ release and absorbance changes also levelled off. Our results suggest that like the fluorescence change, the rate of H^+ release and the rate of the absorbance change level off at high ATP concentration. It therefore appears that the fluorescence change, the absorbance change and the H^+ release are all due to a conformational change which occurs when the irreversibly bound ATP hydrolyzed on the surface of the myosin molecule.

Significance: This work is aimed at gaining a better understanding of the basic mechanism of muscle motility and the control of this motility, phenomena which occur not only in skeletal muscle, but also in such diverse systems as cardiac muscle, arterial smooth muscle, platelets and perhaps within all cells where protoplasmic streaming occurs.

Proposed Course of Research: The most crucial point we will investigate is whether the fraction of subfragment-one (S-1) which neither binds ATP irreversibly or shows the initial Pi burst is, in fact, denatured. It is of importance to make certain that S-1 preparations can be prepared which have very little of this fraction. Purification of the S-1 or a DEAE cellulose column as well as preparation of S-1 with chymotrypsin rather than papain, should help resolve this question. In addition heavy meromyosin and myosin itself will be studied. It is also important to make certain that the fraction of S-1 which does not show the initial Pi burst can still bind ATP reversibly. If it does so, it will be of interest to study the ATPase properties of this fraction both with and without actin present. Even if it is denatured, its properties may aid us in understanding the role of initial Pi burst in undenatured myosin.

Another aspect of this project which requires further study is our finding that H^+ release accompanies the hydrolysis of ATP in the initial Pi burst. It has been reported that the binding of ADP on the ATP analogue AmPPNP also cause release of H^+ and whether this is , in fact, the case will require further investigation.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00411-02 LCB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Reconstitution of E.coli succinoxidase system from sub-components

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

Richard W. Hendler	Head, Sec. on Membrane Enzymology	LCB	NHLBI
Tirumura L. Reddy	Visiting Scientist	LCB	NHLBI

COOPERATING UNITS (if any)

NONE

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Section on Membrane Enzymology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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 small amount of the cytochrome oxidase present in E.coli membranes can saturate the succinate dehydrogenase present to reconstitute succinoxidase activity. The succinate dehydrogenase, complexed with cytochrome b1 bands at an isopycnic density of 1.16 and a major component of cytochrome oxidase has an isopycnic density of about 1.20. When these are recombined to make succinoxidase a new isopycnic species at p=1.18 is formed. The reconstituted succinoxidase is membranous and contains DCCD-sensitive ATPase but little or no outer membrane.

Background: The membrane-associated respiratory chain of E.coli has been solubilized with deoxycholate (DOC) and separated into a succinate dehydrogenase complex and a cytochrome oxidase complex. These two parts can be recombined to yield a functional succinoxidase system. Studies to define the best conditions for isolation of the components, the formation of the integrated system, and the nature of the reconstituted succinoxidase, continue and are described here.

Major Findings: Titration of a fixed quantity of succinate dehydrogenase complex (SDH) with increasing amounts of cytochrome oxidase complex (CO) in order to reconstitute succinoxidase activity (SO) results in a plateau level of maximum SO. The same result is achieved in the alternative titration of CO with SDH. Based on the original amounts present in the membrane, one part of CO can saturate approximately 20 parts of SDH. Therefore, it seems that CO is present at a considerably greater amount that its stoichiometric relation to SDH. This is reasonable because many dehydrogenases introduce electrons to the chain, but apparently only one kind of cytochrome oxidase funnels the electrons to oxygen. A further implication, is that there is no unique structure relation of a particular kind of CO to SDH but that any CO can be linked to the SDH portion.

SDH applied to a column of Sepharose 4B and eluted in 0.1% DOC emerges as a peak with a K_{AV} of approximately 0.63. CO under the same conditions is found to elute with a K_{AV} of 0.74. Both peaks show signs of heterogeneity. The small molecular sizes indicated by these results provide further proof that both SDH and CO are soluble under these conditions.

SDH, freed from DOC, can be banded at an isopycnic density of p=1.16. The band is red in color. This is consistent with our previous findings that SDH is liberated from membranes by DOC in the form of an SDH-cytochrome b_1 complex. CO freed from DOC, is heterogeneous and forms several whitish opalescent isopycnic bands covering the specific gravity range of 1.16 to 1.20. These bands are identified by their ability to form SO. When Sepharose 4B column preparations of SDH and CO are combined to make SO, two isopycnic species are formed. The one of lower buoyant density is also less dense visibly, and it possesses all of the SO (recovery from the gradient is 100%). The heavy contaminant can be separated by prior centrifugation in 10 or 20% sucrose, where it pellets. Alternatively, both the SDH and CO can be initially "cleaned" by prior centrifugation so that only a small amount of the higher density contaminant is formed upon reconstitution. When SDH, cleaned by prior centrifugation, (red band at p=1.16) is combined with Sepharose 4B CO (white opalescent major band at 1.197), the resultant SO appears as a new isopycnic species (reddish band at p=1.18). The original SDH and CO bands are not seen.

The distributions of proteins, ^{55}Fe-labeled cytochromes, ^{3}H-labeled phospholipids, CO, SDH, and SO were followed through the stages of DOC extractions, centrifugations, concentration of extracts and reconstitution, on a particular preparation of E.coli membranes. The final concentrated SDH accounted for 25% of the protein, 31% of the ^{55}Fe, 24% of the phospholipids, none of the CO, 89% of the SDH and 7% of the SO. The final concentrated CO

contained 11% of the protein, 14% of the ^{55}Fe, 21% of the phospholipid, 55% of the CO, 0.3% of the SDH and none of the SO. Concentrated "factor" that enhances SO formation had no SDH, CO or SO; but did have 1.6% of the protein, 0.8% of the ^{55}Fe and 9.6% of the phospholipid. When the SDH, which contained 7% of the SO, was combined with the CO and factor, which had none, 65% of the original SO was recovered. On a different preparation of E.coli membranes, the distribution of protein, SDH, CO, and SO were followed through the Sepharose 4B and isopycnic banding stages. In addition, KDO (marker for the outer membrane), ATPase, and DCCD-sensitive ATPase (marker for energy transduction) were followed. It was found that 1) the isopycnic SDH and CO are virtually free of KDO. 2) The SDH contained 2.6% and the CO, 4.3% of the DCCD-sensitive ATPase. Since the activity requires the union of two components, it is possible that the re-constituted SO accounts for more than the additive 7% present. 3) The extractibility of SDH, factor, and especially CO is quite variable.

Studies with a variety of different membrane preparations show a range of extractibility of CO which results in from 4 to 60% of the original amount subsequently found in the 2 - 0.5% DOC extract. It is interesting to note that with difficult-to-extract preparations much more CO is extracted directly with 2% DOC than can be extracted if the membranes are given a prior extraction with 0.5% DOC, which extracts none of the CO. Among the variables which do not change this result are 1) additional sonication of membranes prior to extraction, 2) sonicating or homogenizing the membranes in DOC during extraction, 3) the state of oxidation or reduction of the respiratory chain, 4) the volume of suspension taken for extraction, 5) treating the membranes with ethanol or butanol prior to extraction, 6) replenishing the 0.5% DOC-extracted membranes with phospholipid, and 7) repetitive freezing and thawing of the membranes. There are some indications that omitting a certain supplement from the growth medium and aging the membranes in liquid nitrogen may influence the extractibility of CO.

Preliminary results with electron microscopy using preparations freed from DOC, indicate that SDH is non-membranous but that CO and SO contain membrane vesicles. In a single attempt at freeze cleavage, no cleavage planes were found. This could be due to the small radius of curvature of the membrane vesicles or to a peculiarity in the particular preparation used for these studies. A combined study using negative staining, thin sectioning and freeze cleavage on the same preparation is contemplated.

Proposed Course: We will try to improve the isolation and characterization of the SDH, CO and "factor" components and to characterize further the nature of the reconstituted SO. Prior to additional electron microscopy, attempts will be made to increase the size of the SO vesicles by a slow removal of DOC during reconstitution.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00412-02 LCB

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Muscle Actin - Acanthamoeba Actin Copolymerization and the Cooperative Interaction Between Actin and Tropomyosin

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

PI: Yeu-Zu Yang, Visiting Fellow — LCB NHLBI

Evan Eisenberg, Head, Section on Cellular Physiology — LCB NHLBI

Edward D. Korn, Chief, Laboratory of Cell Biology — LCB NHLBI

COOPERATING UNITS (If any)

None

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Section on Cellular Physiology

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.3	None

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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) Analogs of random copolymers of muscle actin and Acanthamoeba actin were synthesized. The abilities of copolymers to bind tropomyosin and to activate HMM ATPase were compared with those of homopolymers. It was found that binding between actin and tropomyosin was extremely dependent on Mg^{2+} such that below a certain threshold level of Mg^{2+} no binding occurred. Each actin homopolymer or copolymer had its unique binding isotherm characterized by the threshold Mg^{2+} level and the range in Mg^{2+} concentration within which this all-or-nothing process took place. The narrow range in which this transition occurs indicates that the Mg^{2+}-mediated interaction between the two proteins is extremely cooperative. The activation of HMM ATPase by a copolymer was not significantly different from that of mixed homopolymers, even when the copolymer as well as the mixed homopolymers were completely saturated with tropomyosin. This illustrates that the properties of each parent actin are preserved in the hybrids and expressed independently.

PHS-6040
(Rev. 10-75)

Project Description:

Objectives: It has become increasingly evident that actin is a very well-conserved molecule. Taking advantage of this fact, we have previously proved (Z01 HL 00501-02 LCB) that Acanthamoeba actin and muscle actin copolymerized. In order to characterize the copolymer more thoroughly, this project was carried out by using muscle tropomyosin as a probe, for it was established in our last report (Z01 HL 00412-01 LCB) that at 5 mM $MgCl_2$ and 2 mM ATP, tropomyosin bound to muscle actin but not to Acanthamoeba actin. Of special interest is whether the hybrid molecule resembles both parent molecules or whether the traits of one of the parent molecules is dominant, as the composition of the copolymers varies. Furthermore, in a homopolymer, actin monomers interact cooperatively. Does this cooperativity operate among neighboring actin monomers derived from two different species as remotely-related as amoeba and rabbit? In order to answer these questions, it is important that conditions for co-polymerization be regulated such that the presence of "block" homopolymers be kept at a minimum to assure random distribution of the two kinds of monomers.

Aside from studying the properties of copolymers, the problem of actin-tropomyosin interaction itself is of particular interest because there are contractile systems that lack troponin. The study of actin-tropomyosin interaction may thus reveal how tropomyosin functions in these systems.

Methods Employed and Major Findings: Acanthamoeba actin and muscle actin were prepared as before. Monomeric actins used in copolymerization were obtained by chromatography of each G-actin on a Sephadex G-200 column to eliminate oligomers and aggregates. Copolymer analogs were synthesized by mixing varied proportions of the two monomers at 1 mg/ml and polymerized with 1 mM $MgCl_2$ at 25°. At the end of 4 h, the copolymers were concentrated by ultracentrifugation. Tropomyosin was prepared as before from muscle and repurified by isoelectric precipitation and ammonium sulfate fractionation since traces of contaminating troponin induced binding between actin and tropomyosin under conditions when this would otherwise not have occurred. Binding between actin and ^{125}I-tropomyosin was measured as before by ultracentrifugation. The actin-activated HMM ATPase was determined by the pH-stat method.

The most striking feature of the actin-tropomyosin interaction is our finding that the interaction is extremely dependent on Mg^{2+} concentration so that when the Mg^{2+} concentration exceeds a certain threshold level, an all-or-none response takes place. Each actin homopolymer and copolymer has its characteristic threshold and its range of Mg^{2+} concentrations where such a transition occurs. For example, in the presence of 2 mM ATP, 5 µM actin and 1.25 µM tropomyosin, the transition for muscle actin took place within a very narrow range (4.25 to 5 mM Mg^{2+}) whereas by contrast, for Acanthamoeba actin, it occurred over a wider range and higher concentration (6 to 8 mM Mg^{2+}). In the case of copolymers, the threshold level and the range fell between these two extremes, shifting towards those characteristic of muscle actin as the proportion of muscle actin to Acanthamoeba actin in the copolymer increases; and vice versa. The fact that the binding isotherms for copolymers differ from those of homopolymers indicates that the distribution of the two types of monomers in the copolymers is indeed random.

It is not clear how Mg ions bring about this highly cooperative interaction between actin and tropomyosin; however, we do know that the interaction is an equilibrium rather than a kinetic process. Dissociation of the actin-tropomyosin complex was demonstrated when Mg^{2+} concentration was lowered to below threshold level either by dialysis or by dilution.

Apart from studying the copolymers with respect to their interaction with tropomyosin, their ability to activate the HMM ATPase was investigated. It was found that at 2 mM ATP, 1 mM EGTA, and between Mg^{2+} concentrations of 5 to 9 mM, the activities of the hybrid actin-activated HMM ATPase were not significantly different from those of mixed homopolymers. Therefore, each kind of actin monomer appear to act independently and neither muscle actin nor Acanthamoeba actin behaves dominantly. In addition, if Mg^{2+} concentration was maintained at a level in which total binding of tropomyosin to a copolymer was permissable, the addition of tropomyosin had the same effect on the activity of hybrid actin-activated HMM ATPase as it did on that of the mixed actin-activated HMM ATPase. Again, this illustrates that the properties of each parent actin are preserved in the hybrids and are equally expressed.

Proposed Course of Project: Now that the interaction between actin and tropomyosin is better understood, it would be very interesting to see how troponin subunits affect this interaction. Better and faster methods need to be developed for the preparation of troponin and its subunits as troponin-T, and troponin-I, in particular, decay rapidly. The nature of the Mg^{2+}-mediated cooperative interaction between actin and tropomyosin should be further investigated, initially by studying whether the conformation of each individual protein is affected by changes in magnesium concentrations.

Publications:

Gordon, D., Yang, Y.-Z., Eisenberg, E. and Korn, E. D.: Properties of actin isolated in high yield from Acanthamoeba castellanii. In Goldman, R.D., Pollard, T. and Rosenbaum, J. (Eds.): Cell Motility. New York, Cold Spring Harbor Laboratory, 1976, pp. 493-498.

Yang, Y.-Z., Gordon, D.J., Korn, E. D. and Eisenberg, E.: Interaction between Acanthamoeba actin and rabbit skeletal muscle tropomyosin. J. Biol. Chem. 252: 3374-3378, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 00413-01 LCB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

The Formation of a Ternary Complex: Actin, AMP-PNP, and the Subfragments of Myosin

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

Lois Greene	Staff Fellow	LCB	NHLBI
Evan Eisenberg, Head,	Section Cellular Physiology	LCB	NHLBI

COOPERATING UNITS (if any)

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	None

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

This study was undertaken to characterize the ternary complex composed of actin, AMP-PNP and the subfragments of myosin, subfragment-one (S-1) or heavy meromyosin (HMM). The actin and S-1 bind independently to form a ternary complex with one S-1 molecule binding per F-actin monomer. The binding of actin to S-1·AMP-PNP is relatively strong at low salt, but becomes weaker with increasing ionic strength. The association constant for AMP-PNP to actin·S-1 is $5.4 \times 10^4 M^{-1}$, measured at several ionic strengths. In addition, this study showed that the association constant for the binding of actin to HMM·AMP-PNP was only twice as strong as that obtained with S-1, indicating weak binding of the second head of HMM.

Project Description: Project No. ZO1 HL 00413-01 LCB

Objectives: In muscle cells, the key event in contraction is the interaction of myosin with actin and ATP. There is considerable evidence that the ternary complexes, actomyosin·ADP and actomyosin·ATP, are intermediates of the contractile cycle. In fact, the existence of an actomyosin·ADP ternary complex has been shown to occur both in muscle fibers and in solution with acto·S-1. However, the actomyosin·ATP ternary complex is too short-lived to be studied. Therefore, studies with the non-hydrolyzable ATP analogue AMP-PNP are of interest. Several physiological studies have suggested that AMP-PNP or PPi relax skinned muscle fibers while other more recent studies suggest that many or most of the myosin cross-bridges remain attached to actin in the presence of AMP-PNP. Biochemical studies with isolated actin and myosin have shown that at high salt or low temperature very little ternary complex occurs. However, almost none of these studies have been carried out at low ionic strength at relatively high actin concentration. Therefore in the present study, we investigated first, under what conditions a ternary complex of actin-S-1 and AMP-PNP might be formed and second, what its properties are.

Methods Employed and Major Findings: The formation of the ternary complex was studied using the one-headed and two-headed fragments of myosin, S-1 and HMM, since these are soluble at low ionic strength. The binding studies measuring the dissociation of acto-S-1 or acto-HMM in the presence of AMP-PNP were performed in a Model E analytical ultracentrifuge with UV optics, enabling the direct determination of unbound HMM or S-1. Using this technique, we first demonstrated that a ternary complex of actin, S-1, and AMP-PNP can indeed be formed *in vitro* at saturating concentrations of AMP-PNP. In fact, at 40mM ionic strength the binding constant of actin to S-1-AMP-PNP is 1.1 x $10^5 M^{-1}$ i.e., at a free actin concentration as low as 9μM, half of the S-1-AMP-PNP complex could be observed at experimentally obtainable actin concentration. This is in agreement with *in vivo* data using skinned muscle fibers which suggest that at physiologic ionic strength (0.15 M) most of the cross-bridges are attached to actin filaments.

To be certain that under the conditions of the above experiments, all of the acto-S-1 complex we observed was saturated with AMP-PNP, we determined the binding constant of AMP-PNP to the acto-S-1 complex. Our results show that the binding constant is 5.4 x $10^4 M^{-1}$ and is unaffected by ionic strength. Therefore at an AMP-PNP concentration of 1.5mM, all of the acto-S-1 present will be complexed with AMP-PNP. It is also interesting to note that the binding constant we obtain *in vitro* is very similar to the binding constant of AMP-PNP on the cross-bridge-actin complex observed *in vivo*.

One of the advantages of being able to determine the binding constant of S-1-AMP-PNP to actin, is that it allows an accurate determination of the much stronger binding constant of S-1 to actin, a value of considerable importance which is difficult to measure directly and about which there is considerable controversy in the literature. By using the binding constant of S-1-AMP-PNP to actin as well as the binding constants of AMP-PNP to S-1 and acto-S-1, we can calculate that the binding constatn of S-1 to actin at 0.1 M KCl is $10^7 M^{-1}$. This agrees with several studies in the literature but is much stronger than other reports. We have also been able to demonstrate

that the S-1-AMP-PNP binds to the actin monomers in F-actin at a 1 to 1 molar ratio. Furthermore, the binding appears to be independent over a wide range of S-1 and actin concentrations. There is no evidence for cooperativity occurring as the S-1-AMP-PNP complex binds along the actin filament. We have also found that the turbidity of the acto-S-1 complex is unaffected by the binding of AMP-PNP as long as no dissociation of the actin and S-1 takes place.

Finally, we have begun similar studies with the two-headed myosin fragment, HMM. Our results show that the S-1-AMP-PNP and HMM-AMP-PNP complexes bind to actin with almost the same binding constant. This suggests that the HMM may bind to actin with only one head, the other head binding weakly or not at all.

Significance to Biomedical Research: This work is aimed at gaining a better understanding of the basic mechanism of muscle motility and the control of this motility, phenomena which occur not only in skeletal muscle, but also in such diverse systems as cardiac muscle, arterial smooth muscle, platelets and perhaps within all cells where protoplasmic streaming occurs.

Proposed Course: The plan for this project is to continue investigating the interaction of acto-HMM-AMP-PNP. This is particularly important because it may lead to an understanding of how the two myosin heads interact with actin. The formation of the acto-HMM-AMP-PNP complex will be analyzed to determine whether there is any cooperativity in the binding of HMM to actin, either positive or negative. It is possible that the presence of two heads can interfere with the binding of the HMM to actin when HMM is present in great excess. It will also be of interest to determine the amount of dissociation which occurs as the AMP-PNP concentration is varied. This may give information on how the binding of AMP-PNP to one head affects the binding of the second head to actin.

Additional aspects of this study will be to investigate by electron microscopy the angle of S-1 binding to actin in the ternary complex and to study the effect of the ternary complex on tropomyosin binding. Studies on the effect of temperature on the formation of the ternary complex will also be performed.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HL 00501-03 LCB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Actin of Non-Muscle Cells

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

D.J. Gordon	Staff Associate	LCB NHLBI
E.D. Korn	Chief, Lab. Cell Biol.	LCB NHLBI
J. Boyer	Associate Professor, University of Chicago Medical School	Guest Worker LCB NHLBI

COOPERATING UNITS (if any)

NONE

LAB/BRANCH
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Section on Cellular Biochemistry and Ultrastructure

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.35	2.35	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) We have isolated actin from Acanthamoeba castellanii, human blood platelets, embryonic chick brain, rat liver with particular attention to identifying any species of actin whose polymerization is qualitatively different from that of rabbit skeletal muscle actin. No such species of actin was found. The failure of non-muscle actin to polymerize in crude extracts of these cells must then be attributed to the presence of regulating factors. The identification of such factors and their possible role in cell motility is the subject of future investigation.

PHS-6040
(Rev. 10-76)

Project Description: Project No. Z01 HL 00501-03 LCB

Objectives: It has been observed in several laboratories that actin in non-muscle cells exists largely in the unpolymerized state. Such observations suggest that control of polymerization of actin may play an important role in its function in these cells - a role with no known counterpart in skeletal muscle. We have isolated actin from several sources where such observations have been reported in order to determine whether any of these cells contain a new form of actin instead of, or in addition to, the skeletal muscle type of actin.

Methods Employed: Chromatography on DEAE-cellulose and Sephadex G-150 have been used in the isolation of non-muscle actin. Viscometry has been used to quantify polymerization of actin. ATPase activities have been measured by pH stat or by measuring radioactivity released from γ-^{32}P-labeled ATP. Polyacrylamide gel electrophoresis in dodecyl sulfate has been used to assess purity of actin preparations and isoelectric focusing on polyacrylamide gels to analyze for isoactins.

Major Findings: Last year we found that Acanthamoeba actin differs quantitatively from rabbit skeletal muscle actin in its polymerization behavior, particularly at 5° in the absence of Mg^{++}. However, analysis by van't Hoff plots revealed that this difference was the result of rather small differences in the enthalpies and entropies of polymerization of the two actin species and could not be construed as a major qualitative difference. But when polymerization of actin was attempted in a crude Acanthamoeba homogenate, a significant portion of the actin did not sediment as F-actin and eluted as G-actin on Sephadex G-150. However, when an Acanthamoeba extract containing only that actin which fails to polymerize under conditions normally favoring polymerization (0.1 M KCl, 2 mM $MgCl_2$) was purified by DEAE-cellulose chromatography, the actin-containing fraction polymerized. When this actin was further purified by a cycle of polymerization and by gel filtration, its polymerization was identical to that of Acanthamoeba actin isolated by our usual low-ionic strength extraction procedure. Thus, failure of actin to polymerize in crude Acanthamoeba extracts cannot be due to an unpolymerizable form of actin.

We then examined two cell types where actins with unusual polymerization properties were observed in other laboratories - human platelets and embryonic chick brain. Stracher et al. (Arch. Biochem. Biophys. 167,220-237(1971)) have proposed the existence of two forms of actin in human platelets: Actin I which is irreversibly polymerized except under denaturing conditions and Actin II which does not polymerize in 0.5 M KCl-1 mM CaATP but polymerizes in the presence of Mg^{++} or in the absence of CaATP. In order to refute or confirm these observations, we adapted our Acanthamoeba actin purification method to platelets. We found that purified platelet actin had polymerization properties very close to those of Acanthamoeba actin, differing quantitatively but not qualitatively from muscle actin. In 0.5 M KCl-1 mM CaATP the critical concentration of platelet actin at 5° was >3 mg/ml, in agreement with Stracher's Actin II, but it was noted that rabbit skeletal muscle actin also had a high critical concentration (1 mg/ml) under these conditions. A careful accounting of losses during purification revealed only small losses (≤10%) of actin which

failed to polymerize or depolymerize under the appropriate conditions, which could easily reflect small degrees of denaturation or aggregation. Thus, there is no evidence to suggest an actin in platelets with unique polymerization properties. We also found that purified platelet actin activates the ATPase of muscle heavy meromyosin ~ 80% as effectively as muscle actin, with the same V_{max}, and that it has 1 mole/mole of bound nucleotide.

Bray and Thomas (J. Mol. Biol. 105, 527-548(1976)) reported that only 50% of the actin in embryonic chick brain was polymerizable in crude extracts and that actin purified from such extracts did not polymerize normally. By adapting our method for purification of actin, applied originally to Acanthamoeba actin, to extracts of embryonic chick brains prepared according to Bray and Thomas, we have isolated actin that has the qualitative polymerization properties of muscle actin. Thus, as with Acanthamoeba, the failure of brain actin to polymerize in crude extracts cannot be attributed to a new form of actin. As was also found for platelet actin, chick brain actin is about 80% as effective as muscle actin as an activator of heavy meromyosin Mg^{2+}-ATPase and contains one mole of bound nucleotide per mole of actin monomer.

The general pattern that actins of non-muscle cells are similar to each other quantitatively and qualitatively similar to muscle actin was confirmed by the isolation of actin from rat liver. Using the same procedure, actin was obtained in good yield from rat liver, where it accounts for 1-2% of the total protein rather than 20-30% as in the other sources, and was found to be generally indistinguishable from the other non-muscle vertebrate actins.

All of the actins were analyzed by isoelectric focussing. We confirmed the presence in the vertebrate non-muscle actins of two isoactins, β and γ, with isoelectric points more alkaline than the single α actin component of muscle. On the other hand, Acanthamoeba actin was found to be a single isoelectric species more alkaline than even γ actin.

Proposed Course of Research: Since it now appears that all actins examined have qualitatively similar polymerization properties, the question of why actin does not polymerize in crude cell extracts remains unanswered. It is possible that this could be the effect of a specific control factor which has no counterpart in muscle and which may be an important regulator of non-muscle motility. Identification of such a factor or factors (if they exist) is our next goal.

Publications:

1. Korn, E.D.: Membranes and their association with contractile proteins. In Goldman, R., Pollard, T. and Rosenbaum, J. (Eds.): Cell Motility. Cold Spring Harbor Press 1976, pp. 623-629.

2. Gordon, D.J., Eisenberg, E., Korn, E.D.: Characterization of cytoplasmic actin isolated from Acanthamoeba castellanii by a new method. J. Biol. Chem. 241:4778-4786, 1976.

3. Gordon, D.J., Yang, Y.-Z., Korn, E.D.: Polymerization of Acanthamoeba actin: Kinetics, thermodynamics, and co-polymerization with muscle actin. J. Biol. Chem. 251: 7474-7479, 1976.

PROJECT NUMBER (Do NOT use this space)

HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

Z01 HL 0503-05 LCB

PERIOD COVERED
July 1, 1976 to September 30, 1977

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

Martin Flavin	Head, Sec. on Organelle Biochem.	LCB NHLBI
Takaaki Kobayashi	Visiting Fellow	LCB NHLBI
Todd Martensen	Staff Fellow	LCB NHLBI
Jayasree Nath	Visiting Associate	LCB NHLBI

COOPERATING UNITS (if any)
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LAB/BRANCH
Laboratory of Cell Biology

SECTION
Section on Organelle Biochemistry

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	4	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) Our research deals with the structure, assembly and function of microtubules, which are involved in chromosome segregation, cell motility, the maintenance of cell shape, and the organization of cell surfaces. In the past year we have been studying a post-translational modification of tubulin (the protein building block of microtubules) which we think is an excellent candidate to regulate microtubule assembly or function. This involves the reversible enzymatic addition of a tyrosine residue to the C-terminus of the α-chain of tubulin. We are characterizing the enzymes, substrates and reactions involved. We are also studying tubulin modification in cultured neuronal cells *in vivo*, and have some evidence that brain membrane tubulin is less tyrosylated than cytoplasmic tubulin.

Project Description:

Objectives: We are investigating an enzymic reaction in vertebrate tissues by which a tyrosine residue can be reversibly added to the C-terminus of the α chain of tubulin. Our objective is to discover what this post-translational modification is for. Because it is unprecedented, we suspect that it will prove to have an important function in regulating microtubule assembly or function, either directly or by influencing the partition of tubulin among subcellular compartments.

Methods Employed: Biochemical procedures as indicated under Major Findings.

Major Findings:

a. Tubulin-tyrosine ligase (T. Kobayashi): Last year we reported that the bovine brain enzyme catalyzing tyrosine addition to tubulin could be purified 20-fold by ammonium sulfate precipitation followed by elution from DEAE-cellulose. The yield in the first step could not be increased above 15%. By gel filtration the enzyme had an apparent size of 150,000 daltons in extracts and after salt fractionation, but only 70,000 (now known to be 35,000) when eluted from DEAE; however, addition of crude extract to the latter eluate increased the molecular size again to 150,000. We now know that the enzyme (MW 35,000) exists in brain as a 1:1 stoichiometric complex with an equivalent of dimeric tubulin (MW 110,000). During salt or organic solvent fractionation it is dissociated to the unstable small form, but re-associates with tubulin on removal of salt or solvent. However, if eluted slowly (with a salt gradient) from DEAE, to which free tubulin is more strongly bound, it is obtained in low salt fractions as the 35,000 dalton species. Preliminary titration of the amount of pure dimeric tubulin needed to convert this to the complex indicates that brain contains about 1 enzyme per 150 tubulin molecules.

The enzyme has now been purified 300-fold in 2 steps: a combination batch and column elution of the small form from DEAE followed by elution from an affinity column which has 3 mM tyrosine linked through its carboxyl group via a 3-carbon spacer to an insoluble matrix. Activity can be recovered only if purified tubulin is added both during absorption and elution, to maintain the 150,000 dalton species at all times. The tubulin is then removed by a second DEAE column, again yielding the small form. Similar results were obtained with a 6-carbon spacer and tyrosine linked through its amino group, and when the tyrosine was replaced by aminoethanol, the small form in the absence of tubulin was also hydrophobically absorbed and inactivated, although 95% of the protein passed through. The stickiness of the small form is also indicated by the fact that after acid or acetone precipitation some of the pelleted activity will not redissolve, and by losses a) during dialysis or gel filtration at low salt concentration, or b) by simply incubating with pieces of viscose or cellulose acetate membrane. SDS gel electrophoresis of the 300-fold purified enzyme shows that no discrete band in the molecular weight range ≦35,000 comprises more than 5% of the protein, in accord with our estimate of 1 enzyme per 150 tubulin in brain extracts.

The pH rate profile of the ligase shows a single optimum with half maximum activity at 6.0 and 8.5. The purified enzyme requires ATP specifically; GTP, CTP, UTP, ITP and TTP give <10% as much reaction. Under previous conditions Mg^{2+} was required at a surprisingly high level (12 mM) compared to ATP (2.5 mM); but if salt (or K^+) concentration is increased sufficiently 2 mM Mg^{2+} suffices. Ca^{2+} cannot replace Mg^{2+} and inhibits the reaction, probably not by binding to tubulin since 2 mM saturates the latter (inhibition of assembly) but inhibits ligase only 30% in presence of 5 mM Mg^{2+}. The K_m for tubulin could not be determined and 3-cycle purified tubulin appeared to contain substrate species with different affinities. We would like to compare this K_m value with K_d for dissociation of the 150,000 dalton complex, as this might show whether the latter is an ES complex or an allosteric complex, i.e., in which (a subspecies of) tubulin activates a non-catalytic 35,000 dalton enzyme. We reported last year that 6s tubulin dimers are a substrate, and invertebrate axonemal outer doublet microtubules are not. We still do not know whether 36s rings or microtubules assembled in vitro are substrates, though there are indications that the latter may be in the presence of glycerol.

The first question one wants to ask about tyrosylation is whether it affects the assembly of microtubules in vitro. Unfortunately we do not have authenticated preparations of completely tyrosylated or detyrosylated tubulin. Last year we reported that when ^{14}C-tyrosine had been added to 5 to 25% of the α chains of purified tubulin, and the latter was then put through 3 more cycles of assembly, the label partitioned nearly indiscriminantly between the moieties of tubulin that did and did not polymerize. We now have similar evidence that subunits lacking C-terminal tyrosine can be incorporated into microtubules in vitro. Carboxypeptidase A easily removes any labeled tyrosine introduced by ligase, and in doing so removes some preexisting unlabeled tyrosine (although not as much as expected - see below). Tubulin treated in this way was found to assemble normally. These experiments measured only the final extent of assembly.

Brain tubulin isolated by cycles of assembly is partially tyrosylated (section C) but the exact proportion of α chains with C-terminal tyrosine has been difficult to determine. With large amounts of ligase, the capacity of tubulin to accept tyrosine is determined before and after treating it with carboxypeptidase. Carboxypeptidase is removed before the incubation with ligase by DEAE chromatography, or is inhibited by adding 20 mM β—phenylpropionate. The penultimate glutamate is completely resistant to carboxypeptidase A. Last year we reported that 3-cycle brain tubulin uniformly had an acceptor capacity of 28% before, and 50% after, carboxypeptidase treatment. We now find that these values can vary with different tubulin preparations in the range 15 → 27% to 45 → 56%. When 6s dimeric tubulin was separated from the 36s ring fraction by gel filtration it had the same acceptor capacity as the parental 3-cycle tubulin; thus the failure to tyrosylate 100% after carboxypeptidase is not caused by inability of the rings to serve as substrate. Brain tubulin isolated by DEAE chromatography, and never subjected to warming as in cycles of assembly, gave values of 30 → 34%, suggesting it had relatively little preexisting tyrosine. The ligase-catalyzed reaction is reversible and free tyrosine is released again in the presence of ADP + P_i. Our fractions contain

ATPase, specific activity (nmoles/min x mg) 1.6 in 3-cycle tubulin, 0.16 in DEAE-tubulin, 2.2 in DEAE (first step)-ligase, so an equilibrium might account for the less than complete tyrosylation after carboxypeptidase. But tyrosylation was not increased by using an ATP generating system to prevent the formation of ADP, and when maximally tyrosylated (20%) tubulin was freed of the small molecule fraction and returned to a fresh tyrosylation medium, no more tyrosine was fixed. Successive incubations with ^{14}C- and ^{12}C-tyrosine also showed that in the usual tyrosylation medium there was no exchange of labeled for unlabeled tyrosine in α chains, so that possible exchange reactions do not interfere with this method of assessing the extent to which tubulin is tyrosylated. We assume now either that there is coded heterogeneity in α chains and some forms are not substrates, or that some of the α chains are blocked in some other way or with something other than tyrosine.

Last year we reported evidence that the enzyme detyrosylating α chains in the presence of ADP + P_i was identical to ligase, in that they fractionated together over a 20-fold purification. This has now been confirmed with 300-fold purified ligase. Optimal detyrosylation occurs with 3 mM ADP, 5 mM Mg^{2+} and 10 mM P_i. The rate is reduced to 1/3 with PP_i replacing P_i and to zero with AMP replacing ADP. Caputto's laboratory recently reported that detyrosylation by crude brain extracts did not require ADP and P_i except to reverse an inhibition by the high (12 mM) Mg^{2+} that had previously been used. While this is not correct, it does appear that brain extract contains another detyrosylating carboxypeptidase, distinct from the ligase. We have confirmed their interesting observations that this activity is stimulated by low Mg^{2+} and GTP, which promote tubulin assembly, and inhibited by colchicine, Ca^{2+} or 0.2 M salt, which block assembly. It is also partially inhibited by β-phenylpropionate.

b. Tyrosylation of membrane and cytoplasmic tubulin in cells and extracts (J. Nath): Last year we reported that ligase, when assayed with brain tubulin, could be detected in extracts of every rat tissue examined, the specific activity (nmoles tyrosine fixed/min x mg) ranging from 0.005 in spleen to 0.05 in brain. It now seems likely that this enzyme is absent from invertebrates since it has not been found in lobster ganglia, sea urchin sperm, or eggs before or after fertilization, or in Tetrahymena or yeast. Its absence from invertebrates need not preclude a role in regulating microtubule assembly in vertebrates if one considers, for example, how different systems for regulating muscle contraction have evolved in vertebrates and molluscs. In extracts of cultured neuronal cells we found specific activities of 0.12 for a neuroblastoma, 0.05 for an undifferentiated neuroblastoma-glioma hybrid, 0.06 for the same cells after differentiation, and 0.13 for SV-40 transformed glial cells (transformation involves a decrease in cytoplasmic microtubules and in membrane tubulin). Sympathetic ganglia from 7-day old rats, cultured 2 days with or without nerve growth factor, gave a specific activity of 0.44. NGF induces the synthesis of tubulin and tyrosine hydroxylase, but did not affect the ligase level. We do not know whether the high activity relates to the cell type or the early age of the rats.

Ultimately we want to study the function of tyrosylation in living cells. We have begun to study neuroblastoma cells (neuroblastoma-glioma hybrid NG108-15 kindly provided by Dr. M. Nirenberg) because when they are induced to differentiate there is extensive assembly from a pre-existing pool. We want to

isolate tubulin from cells in the 2 states and compare the extent to which it is tyrosylated. Some results described last year were due to using too dense cell suspensions in which cells died in a few minutes due to acid production. When cells are incubated with ^{14}C-tyrosine in the presence of cycloheximide, tyrosine is fixed almost exclusively into tubulin α chains. Fixation occurs 1/10 as fast as in the absence of cycloheximide, ± a complete mixture of other amino acids, and plateaus after 40 minutes; in the presence of 1 mM colchicine fixation continues longer, especially in differentiated cells. Twenty % of the free, and all of the fixed, tyrosine is inside the cells after 40 minutes incubation. Of the fixed tyrosine which is in the supernatant after homogenization 65-80% is liberated by carboxypeptidase when cells were incubated with cycloheximide, 30% when incubated without. In view of the results on the acceptor capacity of tubulin in vitro (section A) we want to determine whether the ^{14}C fixed in α chains is still in the form of tyrosine, or whether the latter has undergone some modification in cells which does not occur in extracts. There have been some indications that tyrosine is fixed preferentially into a membrane bound fraction of tubulin, but this is no longer certain.

We have now begun to study the tubulin in a crude membrane fraction from rat brain, to determine whether tyrosylation is involved in partitioning tubulin between membrane and cytoplasm. We have not yet been able to purify native tubulin from membranes; it is almost entirely denatured when the membrane pellet is fractionated in sucrose gradients. The crude pellet also inhibits ligase. However when detergent extracts of the pellet are incubated with ligase tyrosine is fixed exclusively into α chains, showing that membrane tubulin can be tyrosylated. The detergent extract itself also has substantial ligase activity. We have tried to estimate the extent to which this solubilized membrane tubulin has C-terminal tyrosine, by determining the maximum capacity to accept tyrosine (as discussed in section A). In crude preparations the amount of total tubulin is estimated by colchicine binding (under our conditions 1 mole of tubulin binds 0.3 mole colchicine). The ratio, moles tyrosine fixed to moles colchicine bound, was found to be 1 to 1.5 for extracts of rat brain, 1.9 for extracts of undifferentiated neuroblastoma, 2.3 for differentiated, and 3.3 for detergent extract of brain membrane tubulin. The result indicates less C-terminal tyrosine in membrane than in cytoplasmic tubulin, but must be confirmed with purified tubulin.

c. Cytoplasmic tubulin - to what extent is it tyrosylated in different cells and subcellular fractions (T. Martensen): The direct approach to determining the extent to which any preparation of tubulin is tyrosylated is to isolate the α chain by SDS gel electrophoresis, digest it with carboxypeptidase, and determine the amount of tyrosine released with an amino acid analyzer. Last year we reported that no tyrosine was found in such a digest of α chain from brain tubulin purified by cycles of assembly. With improved procedures we have now shown that 30-45% of the α chains in that moiety of tubulin obtained by assembly cycles have a C-terminal tyrosine. Lu and Elzinga have meanwhile sequenced a 25 residue C-terminal peptide from a subspecies of α chain obtained after DEAE purification of tubulin, and found a similar amount of C-terminal tyrosine. They also agree that the penultimate residue is a carboxypeptidase-resistant glutamate. The next step would be to compare, in the same brain, the

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total cytoplasmic tubulin, the moiety that assembles, and the 50% that does not assemble from the crude extract; later we would try to study tubulin from less abundant sources such as neuroblastoma. Technical problems have prevented any further progress in this direction.

Last year we reported that the ligase is very specific for tubulin, and other proteins with C-terminal glutamate were not substrates. Neurotransmitters related to tyrosine did not bind to the ligase, but 5 of 6 tyrosine dipeptides were inhibitors and competed with tyrosine. We have now obtained a synthetic octapeptide, gly-glu$_3$-gly-glu$_3$, corresponding to the C-terminal sequence described by Lu and Elzinga, and the corresponding nonapeptide with terminal tyrosine. Neither 35,000 or 150,000 dalton species of ligase catalyzed any exchange or fixation of tyrosine with these, or with gly-glu$_3$-tyr or glu$_3$, and the peptides were only very weak inhibitors compared with tyrosine dipeptides. Carboxypeptidase easily released tyrosine from the penta- and nonapeptides. Trityrosine was not an inhibitor of ligase whereas dityrosine was the most effective dipeptide. The enkephalin tyr-gly-gly-phe-met did not inhibit and denaturated α chain, dissolved in ethylmorpholine acetate after reduction and alkylation in urea, was not an inhibitor or substrate. Small tyrosine peptides mimic tyrosine but larger peptides bind so far to neither tyrosine or tubulin sites.

In analyzing α chains it would be most useful if they could be physically resolved, based either on presence or absence of tyrosine, or on a microheterogeneity which determined whether they could be ligase substrates. Last year we reported that resolution of α chains into 2 peaks on SDS-hydroxylapatite columns was not based on either of these factors. Several investigators have reported electrophoretic resolution of α chains into 2 or 3 components, although usually from axonemal sources. We have found an isoelectric focussing system in which ^{14}C-tyrosylated tubulin is resolved into 1 heavy radioactive band, and 5 unlabeled minor bands of about equal protein content. This is of interest because some minor bands might be non-substrate α chains, or alternatively because microheterogeneity in β chains has not previously been described.

Significance to Biomedical Research: An understanding of what controls microtubule assembly, and the implicit potentiality to modify these controls, is relevant to the following biomedical topics: cell division and growth in relation to mitotic microtubules; regeneration of respiratory cilia, and sperm motility, in relation to axonemal microtubules; mobility and function of cell surface receptors in relation to membrane tubulin or cytoplasmic microtubules; cell differentiation and other attributes of cell shape in relation to cytoplasmic microtubules.

Proposed Course: The ligase is now probably 5% pure after 300-fold purification. Further purification is needed to study the mechanisms of the reactions it catalyzes, about which nothing is known, and may give clues to what controls its activity. The first question here is whether the ligase-tubulin complex which is extracted from brain is an ES complex, or whether it represents an activated form of the 35,000 dalton enzyme. We have no alternative substrate

to test the catalytic activity of the latter, but could study tyrosine binding by equilibrium dialysis. The possible existence of a distinct enzyme that removes tyrosine from tubulin only in the polymeric form would probably give clues to the function and/or regulation of tyrosylation. The variable and limited acceptor capacity of tubulin is an enigma and has also prevented the preparation of tubulin completely tyrosylated or detyrosylated, with which we would like to study in vitro assembly more carefully, i.e., in terms of rates of assembly and disassembly, elongation of microtubule fragments, microtubule ultrastructure, and the presence and distribution of the high molecular weight associated proteins (MAPS). Available evidence on MAPS distribution suggests a higher order periodicity in microtubules which might be conferred by alternating tyrosylated and detyrosylated dimers. It might be possible to prepare fully tyrosylated tubulin by incubating tubulin and ligase with tyrosine in a labile linkage to an insoluble matrix. The limited acceptor capacity of tubulin suggests that: a) there are multiple species of α chain only some of which are substrates for ligase, which can be investigated by further attempts at resolution; or b) that cells, unlike extracts, use something other than tyrosine, or that another enzyme modifies tyrosine, before or after it is bound, into something not susceptible to carboxypeptidase. The second case can be studied by identifying the labeled compound in α chains from cells which have been incubated with ^{14}C-tyrosine. If we could determine the amount of C-terminal glutamate, we could see whether the sum of glutamate and tyrosine account for all of the α chains; this would probably require a non-enzymic sequencing method, and the prior quantitative isolation of a reasonably small C-terminal peptide.

We will continue our attempts to measure directly the amount of tyrosine which carboxypeptidase liberates from α chains isolated from different tubulin preparations, and are presently investigating whether quantitative iodination with $^{125}I_2$ can provide a more sensitive assay for tyrosine. Once we have reproducible results with brain tubulin, we will isolate tubulin from differentiated and undifferentiated neuroblastoma and from other cells and tissues in which there are rapid dimer-polymer transitions, from subcellular fractions such as assembled cytoplasmic and mitotic microtubules and membrane tubulin, from axonemal microtubules of mammalian and invertebrate sperm flagella. We are particularly interested in membrane tubulin because its function is unknown in relation to the effects of antitubulins on cell surface structure and function, and if it can be purified will examine not only its tyrosylation but other properties such as guanine nucleotide and MAPS binding.

The subcellular localization of ligase will be studied, and its distribution between vertebrate and invertebrate tissues. So far only brain cytoplasmic and membrane tubulin have been shown to be substrates, and we plan to investigate axonemal and invertebrate tubulin, as well as cytoplasmic tubulin from other vertebrate tissues.

When we have preparations of tubulin which are completely tyrosylated or detyrosylated we will be able to study in detail the effect of tyrosine on assembly in vitro. But the latter can never be an adequate model for assembly in cells, where assembly is controlled not only in time but in site of initiation and direction of propogation. A specific inhibitor of ligase or a mutant

with modified enzyme might lead us more directly to its function. Meantime we may attempt radioautography of neuroblastoma cells which have fixed tyrosine in the absence of protein synthesis. There is also a possibility of preparing antibodies specific for tyrosylated tubulin, since antibodies have now been isolated which are completely specific for hemoglobin S, although it differs from hemoglobin which does not bind to the antibody in only a single amino acid substitution ($glu_6 \rightarrow val$).

As to possible future projects unrelated to tyrosylation, nothing is known chemically about MAPS, the high molecular weight proteins that coassemble with tubulin in vitro. One might try to isolate these directly with tubulin or tubulin peptide affinity columns, or to determine where they are linked to microtubules by reversible cross-linking, or where they are in cells with specific antibodies.

With respect to flagellar motility we are interested in the so far completely unknown Ca^{2+}-responsive apparatus which mediates steering or tactic responses.

Publications:

1. Raybin, D. and Flavin, M.: Specific enzymatic tyrosylation of the α-chain of tubulin and its possible roles in tubulin assembly and function. In Goldman, R., Pollard, T., and Rosenbaum, J. (Eds.): Cell Motility. New York, Cold Spring Harbor, 1976, pp. 1133-1138.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00504-12 LCB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Plasma Membrane and Phagosome Membrane of Acanthamoeba

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

PI: Anil Verma, Visiting Fellow, Laboratory of Cell Biology, NHLBI

Edward D. Korn, Chief, Laboratory of Cell Biology, NHLBI

Other: None

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Section on Cellular Physiology and Ultrastructure

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	None

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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)

Lipophosphonoglycan, a polymeric glycosphingolipid that comprises about 30% of the plasma membrane of Acanthamoeba castellanii, contains two subunits that can be separated on analytical dodecyl sulfate polyacrylamide electrophoretic gels. We have now developed a preparative procedure for separating the two components by chromatography on polyacrylamide columns in sodium dodecyl sulfate.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Previous work in this laboratory has established that the plasma membrane of Acanthamoeba castellanii consists approximately of one-third phospholipid + sterol, one-third protein and one-third of a novel polymeric glycosphingolipid which we have named lipophosphonoglycan. Lipophosphonoglycan consists of 26% neutral sugars, 3.5% aminosugars, 10% aminophosphonic acids, 14% long chain fatty acids, 13% phytosphingosines and 8% inositol. Last year (Z01 HL 00504-11 LCB) and previous years, we established the general outlines of the structure: inositol seems to be the central moiety to which are linked a ceramide phosphate, the aminosugars, the aminophosphonic acids, and oligosaccharides containing the neutral signs. Lipophosphonoglycan can be separated into two components by electrophoresis in sodium dodecyl sulfate and it seemed probable that the two subunits contain different oligosaccharide substituents. This year we attempted to develop a preparative procedure for the separation of the two subunits which is essential if further meaningful studies of the molecule are to continue.

Methods and Findings: After a number of efforts by many different procedures it was found that lipophosphonoglycan could be separated into its two components by chromatography on polyacrylamide columns in 1% sodium dodecyl sulfate. Other procedures, such as traditional Sephadex column chromatography, even in dodecyl sulfate, were not successful. With these separated components we were able to confirm that the component that migrated more rapidly on dodecyl sulfate electrophoretic gels and was eluted more slowly from the polyacrylamide column (both criteria indicating it is the smaller component) contains glucose, mannose and xylose while the other component contains glucose, mannose and galactose.

Proposed Course of Project: We will attempt to incorporate lipophosphonoglycan and its separate subunits into artificial phospholipid bilayers and measure the effects of the lipophosphonoglycan on the physical properties and permeability of the bilayers. We will attempt to make antibodies to the separated components to determine, if they are not cross-reactive, whether one lies on one side of plasma membrane and one on the other or if both are situated on both sides.

Publications:

1. Dearborn, D. G., Smith, S. and Korn, E. D.: Lipophosphonoglycan of the plasma membrane of Acanthamoeba castellanii. J. Biol. Chem. 251: 2976-2982, 1976.

2. Korn, E. D.: Preface. In Korn, E.D. (Ed.): Methods in Membrane Biology. New York, Plenum Press, 1976, Vol. 6, pp. IX-XI.

3. Korn, E. D.: Preface. In Korn, E.D. (Ed.): Methods in Membrane Biology. New York, Plenum Press, 1976, Vol. 7, pp. XI-XV.

4. Korn, E. D.: Preface. In Korn, E.D. (Ed.): Methods in Membrane Biology. New York, Plenum Press, 1977, Vol. 8, pp. XI-XIV.

5. Korn, E. D. (Ed.): Methods in Membrane Biology. New York, Plenum Press, 1976, Vol. 6, 248 pp.

6. Korn, E. D. (Ed.): Methods in Membrane Biology. New York, Plenum Press, 1976, Vol. 7, 277 pp.

7. Korn, E. D. (Ed.): Methods in Membrane Biology. New York, Plenum Press, 1977, Vol. 8, 368 pp.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HL 00505-11 LCB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Cytology of Acanthamoeba

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: Abdul H. Chagla, Visiting Fellow LCB NHLBI

COOPERATING UNITS (if any)

None

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Laboratory of Cell Biology

SECTION
Section on Cellular Biochemistry and Ultrastructure

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	None

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) The rates of phagocytosis and pinocytosis in Acanthamoeba under several experimental conditions were affected quantitatively to the same degree, suggesting that cellular mechanisms are the same for both events. Increasing phagocytic uptake decreases the amount of pinocytic uptake, but the total volume of uptake remains constant. Pinocytosis is not "saturable" but phagocytosis ceases when about 15% of the cell volume is occupied by indigestable particles. The data suggest that the volume of an internal compartment limits the rate of endocytosis. We examined phagosome membranes with freeze-fracture replication and found an increased density of intramembranous particles in the phagosome membrane as compared to the surface membrane from which it derived. The inrease in particle density occurs after separation of the phagosome from the cell surface. We are attempting to confirm and extend the morphological observations by isolating a pure phagosome membrane fraction in order to compare the protein/lipid ratio with that of the surface membrane. Efforts this year have concentrated on establishing the purity of the isolated phagosome membranes. In the coming year we will focus on elucidation of the cytological mechanism for increase in phagosome intramembraneous particle content, and the analysis of the purified membrane fractions.

PHS-6040

Project Description:

Objectives: To elucidate the structural basis of biochemical and physiological events in the soil ameba, Acanthamoeba castellanii. Our current emphasis is on understanding more about the interrelationships and possible interconversions of internal membrane systems with those of the plasma membrane through biochemical and morphological studies of endocytosing cells and through morphological studies of the membranes with freeze-fracture replication.

Methods Employed: Transmission electron microscopy is being used for the study of fixed and embedded cells and for evaluation of isolated cell fractions. The technique of freeze-fracture replication (also dependent on transmission electron microscopy) is being used for the study of surface and internal membrane morphology.

Phagosomes are being isolated by gradient density centrifugation and their purity is being assayed by standard biochemical procedures.

Major Findings: 1) In Acanthamoeba the rate of membrane uptake accompanying endocytosis is sufficiently great that the ameba must recirculate surface membrane. In continuing studies of the route of this membrane circulation and of membrane interrelationships within the cell we examined phagocytosing Acanthamoeba in thin section and by freeze-fracture replication. Freeze-fracture replication allows morphological distinction between certain kinds of membranes and may also demonstrate changes in membrane organization under different physiological conditions.

We used lipid-extracted baker's yeast as the particle to be phagocytosed. After uptake by the ameba, the membrane surrounding a yeast particle could be identified in the replica because the membrane closely follows the contours of the underlying yeast cell which has a uniquely shaped bud scar. The intramembraneous particle (IMP) density and size distribution were measured on both fracture faces of the phagosome membrane and compared with the same two parameters on plasma membrane fracture faces. The phagosome IMP density was found to be approximately 4 times that of the plasma membrane. Since IMP have been shown by many investigators to be related to membrane protein content, the implication is that phagosome membranes have by some mechanism become more concentrated in protein. Possible mechanisms for this increase in IMP density were explored.

There is no accumulation of particles in the plasma membrane at the site of binding of yeast (i.e., phagocytosed particle) to the cell surface or in the forming phagosome. Therefore it appears that the increase in IMP density occurs after the internalization of the phagosome.

In thin sections phagosomes show evidence of many fusions with small vesicles. By use of the tracer, horseradish peroxidase, some of these vesicles were proved to be pinocytic in origin, and thus should represent addition of membrane similar in composition to the initial enveloping plasma membrane. Vesicles of unidentified origin also fuse with or bud from phagosomes, but no vesicles with very high IMP densities were observed in replicas.

Comparison of the size distribution of IMP in the phagosome membrane and the plasma membrane gave no evidence for appearance of new classes of IMP in the phagosome. In addition, particle-free and low-particle-density vesicles are very numerous in the cytoplasm. These last observations suggest the possibility of a mechanism of particle concentration by preferential removal of lipid from the phagosome membrane, and point to a more complex situation than a simple recirculation of unchanged plasma membrane through the phagosome back to the surface via a defecation vacuole or small vesicle transport.

2) The freeze-fracture observations on intact cells suggest that the protein/lipid ratio in the phagosome membrane should be higher than that of the plasma membrane. In order to obtain biochemical confirmation of the morphological observations described above, Dr. A. H. Chagla has undertaken to isolate a pure phagosome membrane preparation and compare its protein to lipid ratio with that of the plasma membrane. Efforts this first year have been concentrated on establishing criteria for purity of the membrane preparation.

Phagosome membranes are isolated by procedures described by Wetzel and Korn for Acanthamoeba. Amebas are fed latex beads, then homogenized and the phagosomes containing beads are floated free of most other membranes with sucrose density gradient centrifugation. Membranes are dissociated from the latex particles by sonication and collected and washed by centrifugation. Potential contamination of phagosome membranes by other membranes or cell organelles is being assayed in three ways. The first is by electron microscopy of the membrane pellets. The pellets comprise mainly very small closed vesicles of membrane. There is some minor contamination with lipid droplets and glycogen granules. The second is by assay of the pellet for enzymes that are markers for particular membranes or cell organelles. The washed phagosome membranes contain no detectable succinic dehydrogenase activity, RNA, or thiamine pyrophosphatase, indicating no appreciable contamination with mitochondria, rough endoplasmic reticulum, or (perhaps) Golgi membranes. Whereas thiamine pyrophosphatase has been used as a marker for Golgi in other cell types and has an activity of 0.009 μmol/min/mg protein in the whole homogenate of Acanthamoeba, we have not confirmed its usefulness as a Golgi marker in Acanthamoeba. On the other hand, it appears there may be some contamination of the isolated membranes with lysosomal enzymes and with contractile vacuole membrane. We have used alkaline phosphatase as a marker for contractile vacuole membrane, since we have previously shown by cytochemical methods that alkaline phosphatase activity is localized exclusively in the contractile vacuole in Acanthamoeba. We have used acid phosphatase and β-glucosidase as lysosomal enzyme markers. A third approach to detection of contamination is by use of radioactivity labeled amebas. Amebas were grown in the presence of tritiated leucine for 48 hours. The cells were washed, homogenized and centrifuged. A hot supernatant fraction and a hot pellet fraction were mixed separately with two aliquots of cold amebas that had ingested latex beads and the phagosome fractions isolated. From a count of the phagosome fractions we estimate about 12% contamination with membranes at that point. There is additional contamination from the supernatant (soluble) fraction that may be removed with subsequent washings during isolation of membranes from the

phagosome fraction. This approach seems to be the most sensitive assay of contamination and is being pursued. A separate experiment has shown that growing cells with tritiated leucine as precursor for 48 hr gives the following count distribution: 60% of the counts are TCA precipitable, 30% are extractable with lipid solvents, and 10% are soluble. Of the lipid soluble counts, 88% were found in neutral lipids and 12% in phospholipids.

3) Acanthamoeba, with high rates of phagocytosis and pinocytosis of the non-concentrative type, offers favorable experimental material for investigation of similarities and possible differences in these two modes of uptake. We measured phagocytosis by the rate of uptake of latex beads and pinocytosis by the rate of uptake of radioactive inulin and albumin. The effects of the metabolic inhibitors NaN_3, NaCN, NaF, iodoacetate, 2,4-dinitrophenol and cold were found to be identical on both forms of endocytosis. Both endocytic processes were suppressed by inhibitors of aerobic metabolism and low temperature and were not appreciably affected by inhibitors of glycolysis. The cells recovered capacity to endocytose after exposure to all these compounds except 2,4-dinitrophenol, which was irreversibly toxic. Endocytosis and O_2 consumption were measured as a function of temperature. Below 5°C both phagocytosis and pinocytosis ceased; between 9 and 15°C uptake was less than 10% that at 29°C. From 16 to 29°C uptake was a linear function of temperature for both pinocytosis and phagocytosis. Curves for O_2 consumption and endocytosis both showed breaks at about 16°C. Concanavalin A, which can cause agglutination of Acanthamoeba, inhibited both types of endocytosis more than 50% at concentrations as low as 5 μg/2 x 10^5 cells/ml. At these concentrations of Con A and cells, there was only minor agglutination of the cells so that the effect of Con A appears to be one of direct interference with endocytosis. Pinocytosis and phagocytosis were also measured simultaneously in the same cells. Increasing the rate of phagocytosis suppressed pinocytosis, but the combined volume of the two forms of uptake was essentially constant. In contrast, the estimated combined surface intake varied over a two-fold range. These data show no differences between phagocytosis and pinocytosis of the non-concentrative type, and suggest that control of the rate of endocytosis is determined by the volume of an internal compartment. The volume of this compartment, estimated by measuring the volume of latex beads that "saturate" the phagocytic mechanism, amounted to about 500 μm^3 per cell or roughly 15% of the cell volume.

Proposed Course of Project: We will continue with the phagosome membrane isolation and attempt to compare phagosome membrane with plasma membrane with respect to protein to lipid ratio and protein profiles on polyacrylamide electrophoresis. We will seek ways of following specific vesicle fusions and fissions associated with phagosomes in order to understand better possible routes of membrane recirculation.

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00506-02 LCB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Acanthamoeba Myosin II

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

PI: Hiroshi Maruta, Visiting Associate — LCB NHLBI

Edward D. Korn, Chief, Laboratory of Cell Biology — LCB NHLBI

Hana Gadasi, Visiting Fellow — LCB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
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SECTION
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INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.85	1.85	None

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) We have purified from Acanthamoeba castellanii a second myosin ATPase, Acanthamoeba myosin II, which induces syneresis when added to gels formed from F-actin and purified Acanthamoeba gelation factors. This myosin differs in many ways from the first myosin ATPase, Acanthamoeba myosin I, previously isolated from the same organism. Myosin II has a native molecular weight of about 350,000 derived from multiples of a heavy chain of about 170,000 daltons and two different light chains of about 17,500 and 17,000 daltons and is, therefore, a two-headed myosin. Its very low Mg^{2+}-ATPase activity is stimulated only about two times by F-actin even in the presence of Acanthamoeba myosin I cofactor proteins. In contrast, myosin I is a single-headed enzyme of native molecular weight about 180,000 consisting of a heavy chain of 140,000 daltons and light chains of 16,000 and 14,000 daltons, Its Mg^{2+}-ATPase activity is highly activated by F-actin in the presence of cofactor proteins. These, and other significant physical and enzymatic differences indicate that Acanthamoeba myosin I and II are the first known examples of the occurrence of two different myosins in the same cell where they presumably have different functions.

Project Description:

Objectives: In 1975, investigators in other laboratories found that cold extracts of several different cell types, including Acanthamoeba, form solid gels when warmed to room temperature and that these gels would subsequently shrink, expressing fluid, to form a small pellet of which actin is the major component. Last year (see Z01 HL 00506-01 LCB), we purified four relatively low molecular weight proteins (subunit molecular weights of 23,000, 28,000, 32,000 and 38,000) each of which alone forms a gel with F-actin and which together could account quantitatively for the gelation activity of the crude extracts of Acanthamoeba. Furthermore, we showed that gels formed by the interaction of any one of these gelation factors with F-actin would undergo syneresis (shrinkage or contraction) upon addition of a small aliquot of Acanthamoeba extract. We then showed that this activity co-chromatographed on agarose gels with an ATPase that had myosin-like activities but which was separable from the Acanthamoeba myosin previously described in this laboratory. This raised the very interesting possibility that Acanthamoeba may contain two myosins. The major emphasis this year has been on the purification and characterization of this second Acanthamoeba myosin.

Methods Employed and Major Findings: Purification: We found previously that all of the ATPase activity of Acanthamoeba was recovered in three peaks when extracts were chromatographed on Bio-Gel A 15 m. 1) A small peak of activity was eluted in void volume of the column; 2) a large peak of activity was eluted with a K_D of about 0.33; and 3) another large peak of activity was eluted with a K_D of about 0.67. Each of the three peaks of ATPase activity has been examined for the possible presence of myosin-like enzymes. The ATPase in the void volume has none of the properties of a myosin-like ATPase. The third ATPase fraction contains the Acanthamoeba myosin I previously purified. The (K^+,EDTA)-ATPase of the fraction is more active than its Ca^{2+}-ATPase and its Mg^{2+}-ATPase is activated by F-actin. The second peak of activity is several times more active as a Ca^{2+}-ATPase than as a K^+,EDTA-ATPase and has still less Mg^{2+}-ATPase activity. All the ATPase activity in this fraction binds quantitatively to F-actin. Moreover, this is the fraction that was previously shown to induce the syneresis of F-actin-gels. The major large polypeptide component of this second peak of ATPase is about 170,000 daltons. All these properties are those expected of a myosin-like enzyme and therefore, this enzyme, Acanthamoeba myosin II, was purified further, and characterized. The Acanthamoeba myosin II fraction eluted from Bio-Gel A 15 m was incubated with pancreatic RNase and then freed of RNase and RNA degradation products by chromatography on Sephadex G-100. The void fraction from Sephadex G-100 chromatography was then fractionated by affinity chromatography on ADP-agarose to give an essentially homogeneous enzyme.

Characterization of Myosin II: From its behavior on agarose gel, the native molecular weight of the purified Acanthamoeba myosin II is 350,000 daltons. Electrophoresis on dodecyl sulfate-polyacrylamide gels reveals the presence of a single large polypeptide of about 170,000 daltons and a closely spaced doublet that migrates just behind the A-2 light chain of rabbit skeletal muscle myosin

at positions corresponding to approximately 17,500 and 17,000 daltons. The two light chains are widely separated on urea-polyacrylamide electrophoretic gels. The enzyme binds quantitatively to F-actin in the absence, but not in the presence, of ATP and to G-actin-DNase-agarose. F-actin inhibits the Ca^{2+}-ATPase activity of Acanthamoeba myosin II and consistently activates its Mg^{2+}-ATPase, albeit only 1.4 to 2 fold. Actin-activation of the hydrolysis of GTP by Acanthamoeba myosin II was consistently greater (4-fold) than when ATP was the substrate. We found previously that actin-activation of Mg^{2+}-ATPase activity of Acanthamoeba myosin I occurred only in the presence of an additional, partially purified cofactor protein. To test the possibility that the same cofactor protein might also stimulate actin-activation of Mg^{2+}-ATPase activity of Acanthamoeba myosin II, we separated the cofactor protein from ATPase activity. Cofactor protein that was fully competent to allow actin-activation of Acanthamoeba myosin I had no effect on actin-activation of Acanthamoeba myosin II.

In order to test the possibility that Acanthamoeba myosin I might be a proteolytic degradation product of Acanthamoeba myosin II, or that both might be derived from yet a larger native enzyme, we examined the possible effects of including inhibitors of proteolysis in the isolation procedure. Cells were homogenized in the usual buffer with the addition of phenylmethylsulfonylfluoride, p-chloromercuribenzoate and EDTA, each a known inhibitor of one or more proteolytic enzymes, and the extract was chromatographed in the usual way on an agarose column. The ratio and amounts of ATPase activities attributable to Acanthamoeba myosins I and II and the distribution of high molecular weight polypeptides were the same as in a parallel experiment without the inhibitors.

Proposed Course of Project: Better purification procedures will be developed for the isolation of Acanthamoeba myosin I and it will be compared to Acanthamoeba myosin II by several procedures probably including peptide mapping, immunochemical analysis, and N-terminal and C-terminal amino acid analysis in order to establish definitively whether these two proteins are related. We will continue to develop purification procedures for the cofactor protein(s) required for actin-activation of Acanthamoeba myosin I and hope to determine their function.

Publications:

1. Maruta, H. and Korn, E. D.: Purification from Acanthamoeba castellanii of proteins that induce gelation and syneresis of F-actin. J. Biol. Chem. 252: 399-402, 1977.

2. Korn, E. D.: Biochemistry of motility in Acanthamoeba castellanii. In Perry, S.V., Margreth, A. and Adelstein, R. S. (Eds.): Contractile Systems in Non-Muscle Tissues. Amsterdam-Oxford-New York, Elsevier/North-Holland Biomedical Press, 1976, pp. 285-296.

3. Maruta, H. and Korn, E. D.: Acanthamoeba myosin II. J. Biol. Chem., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00507-01 LCB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Interactions Between Spectrin and Actin

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

Esther Reichstein,	Visiting Fellow	LCB	NHLBI
Edward Korn, Chief,	Lab. Cell Biol.,	LCB	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Section on Cellular Biochemistry and Ultrastructure

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	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)

We have sought evidence for the interaction between sheep erythrocyte spectrin and rabbit skeletal muscle actin using viscometry and affinity-chromatography and a direct binding assay. To date results have been inconclusive.

PHS-6040
(Rev. 10-76)

Objectives: The shape changes that the erythrocyte undergoes in vitro is a manifestation of the membrane-bound 'contractile' system of this cell. In order to elucidate the interactions between the proteins comprising this system which give rise to and control shape changes, we have undertaken a study of some properties of sheep erythrocyte spectrin and its interaction with rabbit skeletal muscle actin.

Methods Employed and Findings: Low ionic strength extracts of hemoglobin-free sheep erythrocyte membranes contain both spectrin and actin. Such extracts, like those of human erythrocyte membranes, exhibit a Mg^{2+}-ATPase of low specific activity as measured in a radioassay using γ-labelled ATP^{32}. In contrast to observations with human erythrocytes, such extracts have a much lower Ca^{2+}-ATPase activity.

Spectrin, freed of actin by gel filtration on Sepharose 4B or Biogel A-15 M exhibited a lower ATPase activity than crude extracts and Mg^{2+}-ATPase was recovered in fractions other than those containing spectrin. However, ATPase activity was recovered only in very low yield after gel filtration.

These actin-free spectrin preparations were used in the study of spectrin-actin interactions measured by three methods: a) effects of spectrin on the polymerization of muscle actin as measured by viscosity, b) the effect of spectrin on the actin-activated heavy meromyosin Mg^{2+}-ATPase activity, and c) binding of radioiodinated actin to spectrin as measured by gel filtration. The latter two methods failed to show an interaction; however, viscosity measurements indicated that spectrin and F-actin interact. At low actin concentrations (<10 moles actin/mole spectrin) the specific viscosity of actin was increased by its presence. However this increased viscosity was dissipated by the process of viscometry, while decreases in viscosity at low actin were not observed in all spectrin preparations.

We are now doing experiments using columns of actin bound to DNAase-agarose and actin polymerized in the presence of Sepharose to measure spectrin binding to G-actin and to F-actin.

Proposed Course: Conditions will be sought to stabilize spectrin and quantitative measurements of spectrin-actin interaction will be developed.

Publications: None

ANNUAL REPORT OF THE
LABORATORY OF CELLULAR METABOLISM
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

July 1, 1976 through September 30, 1977

Research in the Laboratory of Cellular Metabolism has in the past dealt with a number of aspects of metabolic control in mammalian cells with emphasis at different times on the mechanisms of action of catecholamines, glucocorticoids, prostaglandins and certain peptide hormones. This portion of the research program is now essentially completely focussed on the enzymes responsible for the synthesis and degradation of cyclic AMP and cyclic GMP, through which many hormones, drugs and other internal and external stimuli influence cellular functions. Work on the control of histamine release from mast cells was terminated this year with retirement of the principal investigator. The continuing study of mechanisms for regulation of cholesterol synthesis has undergone some reorientation with the initiation of a combined biochemical and morphological investigation of cholesterol uptake and removal from cultured human fibroblasts.

1. Cyclic Nucleotide Phosphodiesterases

Most mammalian tissues contain three major types of soluble cyclic nucleotide phosphodiesterases that can be separated chromatographically. E I is an enzyme with a high affinity for cGMP (which also hydrolyzes cAMP) whose activity is increased by a heat-stable protein activator in the presence of calcium. E II also has a high affinity for cGMP but exhibits anomalous kinetics and is sometimes referred to as a cGMP-stimulated cAMP phosphodiesterase. E III is a low K_m cAMP phosphodiesterase. In studies of cultured hepatoma cells completed this year it was shown that the activities of E I and E II were decreased by dexamethasone treatment with no change in E III. Incubation of the cells with dibutyryl cAMP on the other hand did not alter the activities of E I and E II but doubled the activity of E III.

We had previously found that E I which is reversibly activated by calcium plus the protein activator can be irreversibly activated to the same level by controlled proteolysis with chymotrypsin. Activation by chymotrypsin is associated with an apparent decrease in molecular size and an increased susceptibility to thermal inactivation. In collaboration with the Laboratory of Kidney and Electrolyte Metabolism, NHLBI, we have shown that E I is also activated by a heat-labile protein which is released under hypotonic conditions from a particulate fraction of rat renal cortex. The characteristics of the activation induced by the renal protein were similar in several respects to that induced by chymotrypsin. The renal protein, however, also activates E III which in our studies is not activated by chymotrypsin. The physiological role of this type of activation and/or of the renal protein, which may be a lysosomal protease, remains to be defined.

Current phosphodiesterase work is concentrated on the purification and characterization of E I. The calcium-dependent heat-stable protein activator has been purified from rat liver and from bovine brain. These materials will be used for affinity chromatography of the phosphodiesterase as well as for studies of activator structure and interaction with calcium.

2. Adenylate Cyclase

Adenylate cyclase studies this year have again been concentrated in large part on the mechanism by which choleragen activates the enzyme and the interaction of the toxin with ganglioside G_{M1} which serves as its cell surface receptor. For some of the latter work liposomes prepared with lecithin, cholesterol and dicetylphosphate were used as model membranes. Choleragen was bound to and increased the release of trapped glucose from liposomes containing G_{M1} but apparently did not interact significantly with liposomes lacking ganglioside or with those containing G_{M2} or G_{D1a}. The extent of glucose release was dependent on the G_{M1} content of liposomes and the concentration of choleragen. Incubation of G_{M1}-liposomes with anti-G_{M1} serum prevented the release of glucose on subsequent exposure to choleragen. The B protomer of choleragen which is the portion of the molecule that binds to the cellular receptor was as effective as the holotoxin in causing release of glucose from G_{M1} liposomes whereas the A protomer was inactive. Thus, interaction of the ganglioside binding site on the B protomer with G_{M1} in the liposomes, in the absence of other proteins, was apparently responsible for perturbations of the lipid model membrane that were evidenced by glucose release.

Using anticholeragen antibodies and complement to magnify effects on membrane permeability it was shown that the A protomer but not the B protomer or the intact choleragen induced release of glucose from ganglioside-free liposomes. Others have suggested that release of the A protomer from choleragen is required for activation of adenylate cyclase and it appears from our studies that the A protomer must be freed of constraints present in the intact choleragen in order to interact with the liposomal model membranes. The binding of B protomer to the G_{M1} receptor may, in addition to bringing the A protomer into proximity with the cell membrane, cause perturbations of the membrane or the choleragen molecule that promote the interaction with A protomer.

Measurements of tryptophanyl fluorescence were also used to monitor the interactions of choleragen and gangliosides. It was inferred from the fluorescence spectrum of the A protomer that the tryptophanyl residues were located in a nonpolar environment and additional hydrophobic interactions would not be expected to alter the absorption maximum. The fluorescence intensity of the A protomer was, however, increased by all of the gangliosides tested, perhaps a result of relatively non-specific lipid-protein interactions. G_{M1}, but not G_{M2}, G_{M3}, or G_{D1a}, caused a change in the fluorescence spectrum of the B protomer which may be interpreted as indicative of a shift of the tryptophanyl residues to a less polar environment. The alterations in B protomer conformation reflected by the "blue shift" in fluorescence could, when choleragen binds specifically to G_{M1} on a cell surface, promote dissociation of the A protomer or facilitate its interaction with other lipids in the plasma membrane or both.

Although the B protomer is responsible for binding of choleragen to the cell surface receptor, G_{M1}, it is the A protomer which activates adenylate cyclase. Other workers had shown that NAD is required for this reaction but its role was unknown. Based on the hypothesis that choleragen activation of adenylate cyclase is an enzymatic process we looked for catalytic activity of the toxin molecule using NAD as substrate. We were able to demonstrate that choleragen or its A protomer hydrolyzed NAD to yield ADP ribose and nicotinamide activity and described some characteristics of this enzymatic activity. It was suspected that the NAD glycohydrolase activity of the A protomer of choleragen was analagous to that exhibited by the A fragment of diphtheria toxin (Kandel et al., 1974) and represents an abortive reaction in which water rather than a specific second substrate serves as an acceptor for the ADP-ribosyl moiety of NAD. We undertook, therefore, to demonstrate that choleragen does, in fact, possess ADP-ribosyl transferase activity.

Arginine or arginine methyl ester but not a number of other amino acids or their derivatives markedly increased the rate of nicotinamide formation from NAD in the presence of choleragen. After incubation of the toxin with NAD and arginine a product was isolated which contained adenine and arginine in a one to one ratio as would be expected in ADP-ribosyl arginine. All evidence was consistent with the conclusion that it is the guanidino moiety of arginine that acts as the acceptor in the ADP-ribosyl transferase reaction. Based on the assumption that this reaction is a model for the NAD-dependent activation of adenylate cyclase by choleragen it was proposed that the active A protomer of choleragen catalyzes the ADP-ribosylation of an arginine, or related amino acid residue in a protein which is the cyclase itself or is critical to its activation by the toxin. We are now working on purification of the adenylate cyclase system with the goal of demonstrating directly the site of ADP-ribosylation and the role of the ADP-ribosylated protein in the regulation of adenylate cyclase activity.

Since glycopeptide hormones such as thyrotropin and human chorionic gonadotropin (hCG) share with choleragen certain homologies of primary structure it has been suggested that they might activate adenylate cyclase by a similar mechanism. Although we were unable to show that activation of thyroid adenylate cyclase by thyrotropin is dependent on or enhanced by NAD, we found, in collaborative studies with workers in the Department of Medicine, Columbia University, that highly purified preparations of thyrotropin and of hCG possessed NAD glycohydrolase activity. This activity was also present in apparently homogeneous preparations of α and β subunits of hCG and in the asialo or reduced carboxymethylated derivatives. Recombination of the hCG subunits, which restores biological potency, did not, however, enhance NAD glycohydrolase activity. Furthermore, this activity did not co-chromatograph with purified hCG or its α or β subunits. It appeared, therefore, that NAD glycohydrolase activity was not an intrinsic property of hCG or its subunits. Thus, there is no evidence that these glycopeptide hormones activate adenylate cyclase through an NAD-dependent mechanism analagous to that of choleragen.

3. Guanylate Cyclase

In studies of the guanylate cyclase begun last year we found that the soluble enzyme from kidney was markedly stimulated by Co^{++} ion which had no

effect on activity of the particulate cyclase. Incubation of kidney slices with Co^{++} did not, however, increase their cGMP content. Co^{++} activation of the guanylate cyclase was half maximal with 20 μM and maximal with 100 μM (in the presence of 5 mM $MnCl_2$). Incubation of fresh kidney supernatants at 37° for 3 h increased guanylate cyclase activity and abolished Co^{++} activation. In fresh supernatants, increasing assay pH from 5.9 to 9 increased enzyme activity in a linear fashion whereas in the presence of Co^{++} there was a clear maximum at pH 7.3 to 7.5.

To further investigate the nature of the Co^{++} effect we undertook to purify the soluble guanylate cyclase from rat liver which has properties similar in many ways to those of the kidney enzyme. The enzyme activity in fresh supernatants was absolutely dependent on Mn^{++} and neither Co^{++} nor Mg^{++} substituted for or altered the "K_a" for Mn^{++}. Of the ions tested, which included Ca^{++}, Mg^{++}, Ni^{++}, Pb^{++}, Cu^{++}, Zn^{++}, Sn^{++}, Ba^{++}, Fe^{++} and Fe^{+++}, none replaced Co^{++}. The soluble hepatic guanylate cyclase has now been purified about 500-fold using salt fractionation, ion exchange chromatography, gel filtration, density gradient centrifugation and preparative disc gel electrophoresis.

Last year we described an inhibitor of guanylate cyclase present in the supernatant fraction from kidney. A heat-stable inhibitor of relatively low molecular weight has now been found in liver. This material partially purified by chromatography on Sephadex G-25 does not affect guanylate cyclase activity in fresh supernatant but does inhibit the partially purified enzyme. Co^{++} in concentrations that activate the enzyme in fresh supernatant has no effect on the partially purified guanylate cyclase but reverses the effect of the inhibitor. Further purification and characterization of the soluble hepatic guanylate cyclase and the inhibitor is continuing as a necessary preliminary to investigation of the mechanisms for control enzyme activity and the action of Co^{++}.

4. Regulation of Cholesterol Metabolism in Mammalian Cells

Cultured human fibroblasts have been extensively used (in this laboratory and elsewhere) for studies of cholesterol synthesis and the defects in negative feedback regulation that are expressed in cells from patients with Type II hyperlipoproteinemia (familial hypercholesterolemia). During the past year investigation of the effects of digitonin and of Fe^{++} or Mn^{++} ions on cholesterol synthesis has continued but most effort has been directed toward the initiation of studies of the mechanisms by which cholesterol is taken up by and removed from cells. Both biochemical and morphologic techniques are being used to evaluate the role of lysosomes in formation of the cholesterol-containing lipid droplets that accumulate in cells on exposure to certain lipoproteins. For this study it was necessary to devise a sensitive, relatively rapid procedure for quantification of changes in cellular free and esterified cholesterol. In the method developed, free cholesterol is oxidized by cholesterol oxidase and the peroxide formed reacts, in the presence of peroxidase, with p-hydroxyphenylacetic acid to form a stable fluorescent dimer.

To control experimental conditions insofar as possible for these studies human skin fibroblasts were grown (for the first time that we know of) in a completely chemically defined medium. When the cells were then incubated with human low density lipoproteins their free cholesterol content was increased ca. 100% and cholesterol ester content was increased ten-fold. In human fibroblasts grown with medium containing serum exposure to low density lipoproteins caused little change of cholesterol content. The cells grown in serum-free medium accumulated numerous lipid inclusions during the first 24 h of incubation with low density lipoprotein. Examination of these cells using polarization microscopy during the subsequent 24-h period revealed an increasing number of inclusions that exhibited anisotropic birefringence (maltese cross) typical of the cholesterol ester deposits found in atherosclerotic lesions. Having established experimental conditions under which the fibroblasts can be induced to take up lipoproteins rapidly and increase their cholesterol content dramatically, we are now completing work on the methodology for monitoring the lysosomal enzyme content in these cells biochemically and morphologically (using fluorescence microscopy).

The fibroblasts that have been "loaded" with cholesterol are also being used to study the removal of cholesterol from cells. It has been suggested that high density lipoproteins may be important in this process and in preliminary experiments the addition of high density lipoprotein appeared to accelerate cholesterol efflux. This system should provide an excellent opportunity to define the mechanisms for control of cholesterol transport into and out of cells and to further our understanding of the evolution of the cholesterol-containing inclusions that are a characteristic part of the atherosclerotic lesion.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00601-08 CM

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Regulation of Sterol Synthesis in Mammalian Cells Grown in Culture

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

PI: J. Avigan Research Chemist CM NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Cellular Metabolism

SE

INSTITUTE AND LOCATION

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.4	0.7	0.7

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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)

Studies were carried out in human fibroblasts grown in culture on the effects of hormonal factors on sterol synthesis and cellular cholesterol content and on the stimulation of cholesterol synthesis by digitonin and by salts of Fe and Mn. The changes in subcellular pools of free and esterified cholesterol under the influence of environmental factors are being investigated.

Objectives and Background

In addition to the objectives stated last year (Z01 HL 00601-07 CM) we are presently attempting to determine whether agents that stimulate cholesterol synthesis (insulin, digitonin, lipid-free medium) cause, under steady state conditions, a change in intracellular cholesterol concentration, or whether the increase in synthesis is balanced by diminished intake of exogenous cholesterol and/or increased egress.

Methods

Cell cultures are grown as described previously. Assays of cholesterol synthesis and of HMGCoA reductase activity are done by standard procedures. Analysis of free and esterified cholesterol is also carried out in some experiments. The method used is described in another report (Z01 HL 00612-01 CM). In addition, a method with potentially greater sensitivity is being developed. It depends on the enzymatic oxidation of cholesterol with the formation of H_2O_2, oxidation of $Na^{125}I$ to $^{125}I_2$ by the H_2O_2 and assay of the free radioactive iodine. Optimal conditions have been determined for the enzymatic generation of iodine by H_2O_2 in the presence of peroxidase and for its quantitative extraction and assay. The method should be useful in following all oxidation reactions that produce H_2O_2, e.g., in the assay of glucose in picomolar concentrations.

Findings

1. In a preliminary study, the intracellular cholesterol concentration did not seem to be significantly affected by conditions that increased cholesterol synthesis (insulin, serum-free medium, digitonin). The cholesterol balance may therefore be maintained by changes in cholesterol transport.

2. The increase in cholesterol synthesis caused by treatment of fibroblasts for 10 min with digitonin, followed by a 24-hr incubation with a digitonin-free medium, was associated with increased HMGCoA reductase activity. When the 24-hr post-incubation was done in the presence of fetal calf serum, the preceding digitonin treatment also caused a stimulation of sterol synthesis as compared with serum-treated controls. Binding of ^{125}I-LDL was increased, rather than diminished, in the digitonin-treated cells; therefore the mechanism of action does not seem to depend on inhibition of interaction of cells with LDL.

3. Incubation of skin fibroblasts for 4-24 hr with low concentrations of Fe^{++} or Mn^{++}, especially in a serum-free medium, greatly stimulated incorporation of acetate into cholesterol, measured after removal of the above medium. The maximal effect of Fe^{++} was found at a concentration of approximately 3 µM. The stimulation was totally prevented by transferrin. Fe^{++} did not affect the binding of ^{125}I-LDL or the activity of HMGCoA reductase.

Significance to Biomedical Research

Studies on the environmental effects on cellular cholesterol concentration and on cholesterol transport into and out of the cell may promote understanding of conditions contributing to atherosclerosis.

Proposed Course

It is proposed:

1. to further investigate the effects of insulin and other hormones on the concentration of free and esterified cholesterol in fibroblasts.

2. to develop a sensitive assay for cholesterol in a coupled oxidation using labeled iodine as a measure of the substrate oxidized.

3. to determine the mechanism of stimulation of acetate incorporation into sterols by Fe and Mn salts (an effect on the rate of sterol synthesis or an effect in acetate uptake or metabolism).

4. to study by subcellular fractionation and microscopic examination of whole cells (the latter in cooperation with Dr. H. Kruth) shifts in the free and esterified cholesterol pools effected by medium composition of cell culture.

Publications

1. Avigan, J.: Cultured human fibroblasts in the study of atherosclerosis. In Atherosclerosis: Metabolic Morphologic and Clinical Aspects. Adv. Exper. Med. Biol. 82, p 724, Plenum Press, New YOrk, 1977.

2. Avigan, J.: Studies on the effects of hormones on cholesterol synthesis in mammalian cells in culture. In Cholesterol Metabolism and Lipolytic Enzymes, J. Polonovski, Ed., Masson Publ. U.S.A. Inc., New York, 1977, pp 1-12.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 00603-03 CM

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Regulation of Cyclic Nucleotide Phosphodiesterase Activity

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

PI:	V. C. Manganiello	Medical Officer (Research)	CM NHLBI
OTHER:	Joel Moss	Research Associate	CM NHLBI
	Peter S. Ross	Staff Associate	CM NHLBI
	Martha Vaughan	Chief, LCM	CM NHLBI
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COOPERATING UNITS (if any)
None

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.6	1.3	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)

The objectives of this project are to study those factors which regulate cyclic AMP and cyclic GMP phosphodiesterase activities. Three classes of cyclic nucleotide phosphodiesterases have been partially purified from rat liver and HTC hepatoma cells. E-I exhibits classical Michaelis Menten kinetics and a high affinity for cGMP. E-I is activated by Ca^{++} plus a heat-stable protein, by chymotrypsin, and by a heat-labile protein from renal cortex. Treatment with dexamethasone reduces the activity of E-I, and does not alter the activity of the heat stable protein. E-II exhibits positively cooperative kinetics, is inactivated by chymotrypsin and not altered by the renal activator. Interraction of E-II with cGMP leads to enhanced susceptibility to chymotryptic inactivation and thermal inactivation. Treatment with dexamethasone leads to a decrease in the activity of E-II. E-III exhibits a high affinity for cAMP and is activated by the renal activator. Incubation of HTC cells with dibutyryl cAMP leads to a 2-fold increase in E-III activity with no change in either E-I or E-II activity or the activity of the heat stable protein activator.

Work reported under Project #ZO1 HL 00608-03 CM has been incorporated into this project.

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Project Description:

Objectives. To study the structural and regulatory properties of cyclic nucleotide phosphodiesterases in rat liver and fat cells, and HTC hepatoma cells.

Methods. Phosphodiesterase activity was measured by methods previously described in this laboratory. The enzymes were partially purified by standard techniques.

Major Findings. Chromatography of 100,000 x g supernatant fractions from rat liver or HTC hepatoma cells on DEAE or DEAE Biogel columns separated 3 general classes of phosphodiesterases, named E-I, E-II and E-III in reference to their order of elution from the columns.

The E-I class exhibited normal Michaelis-Menten kinetics and a relatively high affinity for cGMP. As reported last year the activity of E-I could be markedly stimulated (at 1 μM cGMP as substrate) by Ca^{++} and a heat stable protein activator and by chymotrypsin. Both Ca^{++} and the protein activator and chymotrypsin activated the enzyme to the same extent; after activation by chymotrypsin, the activity of the phosphodiesterase was not further increased by Ca^{++} and the protein activator. Both types of activation produced similar changes in the kinetic properties of E-I. Although the activation produced by Ca^{++} and the protein activator could be reversed by EGTA, EGTA did not affect the chymotrypsin-activated enzyme. The presence of Ca^{++} and the protein activator did not prevent chymotrypsin induced activation of the phosphodiesterase. Activation produced by chymotrypsin was associated with an apparent decrease in molecular size (as evidenced by sucrose density gradient experiments) and increased susceptibility to thermal inactivation at 48°C.

The E-I phosphodiesterase from rat liver was also activated by a heat-labile protein released under hypotonic conditions from a particulate fraction of rat renal cortex. The characteristics of the activation induced by the renal protein were similar, in several respects, to that induced by chymotrypsin. We suggest that activation of E-I may serve as a model for this type of endogenous regulation of phosphodiesterase activity.

The heat-stable activator from rat liver is a low m.w. protein (m.w. of 12-15,000 based on sedimentation equilibrium studies and SDS disc gel electrophoresis). In preliminary studies, we have found that the amino acid composition of the liver activator exhibits some similarities to published amino acid analyses of similar activators from bovine brain and heart and porcine brain.

Administration of dexamethasone to intact rats or incubation of HTC cells with dexamethasone for 36-48 hr produced a reduction in the activity of E-I. Dexamethasone treatment did not alter the activity of the heat-stable activator.

The second phosphodiesterase eluted from DEAE or DEAE Biogel columns appeared to prefer cGMP as substrate and exhibited kinetics suggestive of "positive cooperativity". cGMP is also the preferred effector for this enzyme (the enzyme is often referred to as a "cGMP-stimulated cAMP phosphodiesterase"). As reported last year, from experiments with a number of cyclic nucleotide and related compounds, we suggested that the specificities of the substrate sites and effector sites were similar. Increasing concentrations of theophylline inhibited the hydrolysis of cGMP and the cGMP-stimulated cAMP hydrolysis to the same extent. E-II was inactivated by chymotrypsin; at concentrations that enhanced hydrolysis of cAMP, cGMP increased the chymotrypsin induced inactivation of E-II. Over the same concentration range, cGMP increased susceptibility of E-II to thermal inactivation at 48°C. Although inactivated by chymotrypsin, the E-II from liver was not inhibited by the chymotrypsin-like activator from the renal cortex.

Incubation of HTC cells with dexamethasone or administration ofdexamethasone to rats in vivo caused a reduction in E-II activity.

The third type of phosphodiesterase class eluted from DEAE or DEAE Biogel columns exhibited a high affinity for cAMP. Incubation of HTC cells with N^6,O^2-dibuturyl cAMP plus 3-isobutyl-1-methylxanthine for 14 hr increased the activity of E-III by about 100%, without affecting the activities of the E-I and E-II enzyme or the heat stable protein activator. E-III was inactivating incubation at 30° with chymotrypsin. Incubation of E-III (eluted from DEAE-Biogel columns) with the renal activator produced a marked increase in cAMP phosphodiesterase activity.

Significance to Heart and Lung Research:

Regulation of cAMP and cGMP content is of undoubted importance in the metabolism of virtually all types of cells and tissues.

Proposed Course:

1. Purification and characterization of the phosphodiesterases with special emphasis on the E-I enzyme and the factors which influence its activity, chymotrypsin, the renal activator and Ca^{++} and the protein activator.

2. Examination of the relationship (if any) between the several forms of phosphodiesterase.

3. Continuation of studies of the regulation (e.g., hormonal) and properties of the cyclic nucleotide phosphodiesterases.

Publications:

1. Vaughan, M.: Regulation of cyclic AMP phosphodiesterase activity. In Dumont, J. E., Brown, B. L., and Marshall, N. J. (Eds.): Eukaryotic Cell Function and Growth. Regulation by Intracellular Cyclic Nucleotides. New York, Plenum Press, 1976, pp 195-202.

2. Manganiello, V. C., and Vaughan, M. Activation and inhibition of fat cell adenylate cyclase by fluoride. J. Biol. Chem. 251:6205-6209, 1976.

3. Lovell-Smith, C. J., Manganiello, V. C., and Vaughan, M.: Solubilization and characterization of hormone-responsive phosphodiesterase activity of rat fat cells. Biochim. Biophys. Acta 497:447-458, 1977.

4. Ross, P.S., Manganiello, V.C., and Vaughan, M.: Regulation of cyclic nucleotide phosphodiesterases in cultured hepatoma cells by dexamethasone and $N^{6},O^{2'}$-dibutyryl adenosine 3':5'-monophosphate. J. Biol. Chem. 252:1448-1452, 1977.

5. Moss, J., Manganiello, V.C., and Vaughan, M.: Substrate and effector specificity of a guanosine 3',5'-monophosphate phosphodiesterase from rat liver. J. Biol. Chem., 1977, in press.

6. Vaughan, M., Manganiello, V.C., Moss, J., and Ross, P.S. Regulation of cyclic nucleotide phosphodiesterase activity. U.S.-U.S.S.R. Joint Symposium on Myocardial Metabolism, May 9-11, 1977, Williamsburg, Va., in press.

PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00606-06 CM

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Cyclic Nucleotide Metabolism in Cultured Cells

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

PI:	J. Moss	Research Associate	CM NHLBI
OTHER:	V. C. Manganiello	Medical Officer (Research)	CM NHLBI
	M. Vaughan	Chief, LCM	CM NHLBI
	P. H. Fishman	Research Biochemist	DMN NINCDS
	R. O. Brady	Chief, DMN	DMN NINCDS
	J. C. Osborne, Jr.	Staff Fellow	MD NHLBI
	B. Brewer, Jr.	Chief, MDB	MD NHLBI
	R. L. Richards	Research Investigator	DI WRAIR
	C. R. Alving	Research Investigator	DI WRAIR

COOPERATING UNITS (if any)

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LAB/BRANCH

Laboratory of Cellular Metabolism

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INSTITUTE AND LOCATION

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.8	1.1	1.7

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Choleragen and A protomer catalyzed the hydrolysis of NAD to ADP-ribose and nicotinamide. The protein with NAD glycohydrolase activity migrated with choleragen on polyacrylamide gels and chromatographed with the A protomer on BioGel P60 columns. NAD hydrolysis was increased in acetate and phosphate buffers, and enhanced >10-fold by dithiothreitol in high concentration. NADP was hydrolyzed slowly by choleragen. Analogues of NAD inhibited the glycohydrolase activity, adenine being the most effective. Formation of nicotinamide from NaD was enhanced by L-arginine and guanidine; L-citrulline was ineffective. The product formed after incubation of [adenine-U-^{14}C]NAD and [^{3}H]-L-arginine with choleragen contained ^{14}C and ^{3}H in the 1:1 ratio expected of ADP-ribose-L-arginine. These results are consistent with choleragen possessing ADP-ribosyltransferase activity with arginine serving as acceptor. Activation of adenylate cyclase may involve the ADP-ribosylation of an arginine in an acceptor protein, which is either the cyclase itself or is involved in cyclase activation.

170

PHS-6040
(Rev. 10-76)

Project Description:

Objectives. To study control of cyclic AMP and cyclic GMP metabolism in cultured cells. The interaction of choleragen with its ganglioside G_{M1} surface receptor, which was investigated previously in transformed cells, was studied in artificial liposomal model membranes. The mechanism of activation of adenylate cyclase by choleragen was studied in an in vitro system, and in normal and transformed fibroblasts.

Methods. Measurement of NAD glycohydrolase and ADP-ribosyltransferase by published methods; ganglioside labeling, binding, synthesis and degradation by methods published or in press. Adenylate cyclase and phosphodiesterase by published methods, choleragen binding and labeling by published procedures.

Major Findings.

(1) Enzymatic activity of choleragen. Choleragen and the isolated A protomer catalyzed the hydrolysis of NAD to ADP-ribose and nicotinamide. The protein with NAD glycohydrolase activity migrated with choleragen on polyacrylamide gels, and chromatographed with the A protomer on Bio-Gel P-60 columns. NAD hydrolysis was proportional to choleragen concentration, increased markedly in acetate and phosphate buffers, and enhanced over 10-fold by dithiothreitol in high concentration. The Michaelis constant for NAD was about 4 mM for both choleragen and the A protomer. NADP was hydrolyzed only slowly by choleragen. Analogues of NAD inhibited the glycohydrolase activity, adenine (k_i=3 mM) being the most effective; significant inhibition was noted with adenosine, AMP, ADP-ribose, nicotinamide, nicotinamide mononucleotide and NADP. When compared at 20 mM AMP was a more effective inhibitor than GMP; IMP and CMP did not inhibit the NADase activity.

Formation of nicotinamide from NAD in the presence of choleragen was enhanced by L-arginine. D-arginine was at least as effective as L-arginine, and L-argininemethylester was considerably more potent than either of them. Guanidine also increased nicotinamide production. L-citrulline, L-lysinemethylester and L-histidine were ineffective. Incubation of [adenine-U-^{14}C]NAD with L-argininemethylester or L-arginine and choleragen resulted in the formation of new compounds which contained ^{14}C, reacted with ninhydrin, and quenched the background fluorescence of plates viewed in ultraviolet light. The reaction product formed after incubation of [adenine-U-^{14}C]NAD with [^{3}H]-L-arginine with choleragen, contained ^{14}C and ^{3}H in the 1:1 ratio expected of ADP-ribose-L-arginine. Those results are consistent with choleragen possessing ADP-ribosyltransferase activity with arginine serving as the acceptor.

Although these enzymatic reactions are only models for the cellular actions of choleragen, they clearly establish that the protein has a catalytic site with strict substrate specificities. This site(s), which is responsible for the NAD glycohydrolase and ADP-ribosyltransferase activities, resides on the A protomer, which is responsible for the

activation of adenylate cyclase. It is therefore logical to assume that activation of adenylate cyclase by choleragen involves the ADP-ribosylation of an arginine or similar amino acid residue in an acceptor protein. The identity of the ADP-ribosylated protein and its role in adenylate cyclase activation remains to be determined.

(2) Interaction of choleragen and its subunits with liposomes. ^{125}I-labeled choleragen was bound to liposomes containing ganglioside G_{M1}, but not in large amounts to liposomes containing G_{M3}, G_{M2}, or G_{D1a}. Choleragen, in the absence of other protein, increased release of trapped glucose from liposomes containing G_{M1}, but had no significant effect on release of glucose from liposomes without ganglioside or those containing G_{D1a} or G_{M2}. The extent of glucose release was dependent on G_{M1} content and choleragen concentration. Prior incubation of G_{M1}-liposomes with anti-G_{M1} serum prevented the choleragen-dependent release of trapped glucose. Addition of anticholeragen antibodies and complement to G_{M1}-liposomes previously incubated with choleragen markedly increased the amount of glucose released.

The B protomer of choleragen was as effective as choleragen in causing release of trapped glucose from liposomes containing G_{M1}. The A protomer did not release glucose from G_{M1}-liposomes. Neither choleragen nor the A or B protomers caused release of trapped glucose from glycolipid-free liposomes. Anticholeragen antibodies and complement, however, caused release of trapped glucose from ganglioside-free liposomes previously incubated with the A protomer but not from those incubated with the B protomer or choleragen. These results suggest that the A protomer, freed of the constraints present in the intact choleragen molecule, can interact with the liposomal model membrane system. To what extent the alterations in the liposomal lamellae induced by choleragen and G_{M1} resemble those induced in the plasma membrane remain to be defined. The liposomal membranes, however, may serve as a useful model for studying receptor-choleragen interaction.

Significance to Heart and Lung Research:

The pulmonary and cardiovascular systems are under the constant control of hormones, prostaglandins and other humoral agents. The effects of many of these compounds are mediated through cell surface receptors which alter cyclic nucleotide synthesis, degradation and localization within the cell. By the use of cultured cells, grown in a rigidly controlled environment, and model systems, we can isolate and define the mechanisms by which hormones, other agents, and their receptors control cyclic nucleotide metabolism in the cell and eventually in the more complex pulmonary and cardiovascular systems.

Proposed Course:

The mechanism of activation of adenylate cyclase by choleragen and hormones will be investigated in normal and transformed fibroblasts.

Model systems for the study of the interaction cell surface receptors with choleragen and hormones will be developed to help further understand the metabolic regulation of normal and transformed cells by their environment.

Publications:

1. Fishman, P. H., Moss, J., and Vaughan, M. Uptake and metabolism of gangliosides in transformed mouse fibroblasts: relationship of ganglioside structure to choleragen response. J. Biol. Chem. 251:4490-4494, 1976.

2. Moss, J., Fishman, P. H., Richards, R. L., Alving, C. R., Vaughan, M., and Brady, R. O. Choleragen-mediated release of trapped glucose from liposomes containing ganglioside G_{M1}. Proc. Natl. Acad. Sci. USA 73:3480-3483, 1976.

3. Manganiello, V. C., Lovell-Smith, C.J., and Vaughan, M. Effects of choleragen on hormonal responsiveness of adenylate cyclase in human fibroblasts and rat fat cells. Biochim. Biophys. Acta 451:62-71, 1976.

4. Moss, J., Manganiello, V. C., and Vaughan, M. Hydrolysis of nicotinamide adenine dinucleotide by choleragen and its A protomer: Possible role in the activation of adenylate cyclase. Proc. Natl. Acad. Sci. USA 73:4424-4427, 1976.

5. Moss, J., Richards, R. L., Alving, C. R., and Fishman, P. H. Effect of the A and B protomers of choleragen on release of trapped glucose from liposomes containing or lacking ganglioside G_{M1}. J. Biol. Chem. 252:797-798, 1977.

6. Moss, J., Osborne, J. C., Jr., Fishman, P. H., Brewer, H. B., Jr., Vaughan, M., and Brady, R. O. Effect of gangliosides and substrate analogues on the hydrolysis of nicotinamide adenine dinucleotide by choleragen. Proc. Natl. Acad. Sci. USA 74:74-78, 1977.

7. Moss, J., and Vaughan, M. Mechanism of action of choleragen. Evidence for ADP-ribosyltransferase activity with arginine as an acceptor. J. Biol. Chem. 252:2455-2457, 1977.

8. Fishman, P. H., Moss, J., and Manganiello, V.C. Synthesis and uptake of gangliosides by choleragen-responsive human fibroblasts. Biochemistry 16:1871-1875, 1977.

9. Moss, J., Manganiello, V.C., and Fishman, P.H. Enzymatic and chemical oxidation of gangliosides in cultured cells: Effects of choleragen. Biochemistry 16:1876-1881, 1977.

10. Vaughan, M. Effects of choleragen and fluoride on adenylate cyclase. In Dumont, J.E., Brown, B.L., and Marshall, N.J. (Eds.): Eukaryotic Cell Function and Growth. Regulation by Intracellular Cyclic Nucleotides. New York, Plenum Press, 1976, pp. 113-124.

11. Moss, J., de Mello, M. C., Vaughan, M., and Beaven, M. A.: Effect of salicylates on histamine and L-histidine metabolism. Inhibition of imidazoleacetate phosphoribosyl transferase. J. Clin. Invest. 58: 137-141, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00607-04 CM

PERIOD COVERED
July 1, 1976 through September 30, 1977

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:	S.-C. Tsai	Research Chemist	CM NHLBI
OTHER:	M. Vaughan	Chief, LCM	CM NHLBI
	V.C.Manganiello	Medical Officer (Research)	CM NHLBI

COOPERATING UNITS (if any)

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NHLBI, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

(1) Soluble (100,000 x g supernatant) guanylate cyclase from guinea pig kidney and liver was activated 3-4 fold by 0.1-0.2 mM Co^{++}. None of a number of other metal ions substituted for Co^{++}. Basal activity increased linearly as assay pH increased from 5.9-9; in the presence of Co^{++} activity was optimal from pH 7.3-7.8. Incubation of fresh supernatants at 37° or dialysis at 0°C increased cyclase activity and decreased the magnitude of cobalt activation. After elution from DEAE Bio-Gel columns, the enzyme was not activated by Co^{++}. (2) Kidney and liver supernatants contain a heat stable dialyzable inhibitor of guanylate cyclase. This material elutes from Sephadex G-25 columns with a K_d of 0.6; it does not affect the fresh soluble cyclase but does inhibit the enzyme after dialysis or elution from a Bio-Gel P-100 column. Inhibition is proportional to the amount of G-25 material added and is completely reversed by Co^{++}. Co^{++} activation of fresh supernatant enzyme may be related to Co^{++} counteracting the effect of the heat stable inhibitor. (3) Guanylate cyclase has been purified approx. 500-fold from rat liver supernatant using ammonium sulfate fractionation, anion exchange, gel filtration and affinity chromatography, sucrose density gradient centrifugation and preparative disc gel electrophoresis.

PHS-6040

Objectives:

To purify guanylate cyclase and elucidate the mechanisms that regulate its activity.

Methods:

Guanylate cyclase is assayed in a system containing 1 mM $\alpha^{32}P_2$GTP, 5 mM $MnCl_2$, 1 mM cGMP, 6 mM theophylline and Tris Cl buffer, pH 7.4. ^{32}P cGMP was purified through columns for radioassay. The cyclase from supernatant of tissue homogenates is purified using salt fractionation and ion exchange, gel and affinity chromatography.

Major Findings:

Renal guanylate cyclase and cyclic nucleotide content. Guanylate cyclase activity and cyclic nucleotide content were studied in individual slices prepared from halved guinea pig kidneys. Basal guanylate cyclase activity, assayed in homogenates or in particulate fractions (100,000 g x 1 h), and the tissue content of cGMP and cAMP were greater in the inner than in the outer (entirely cortical) slices. The fraction of guanylate cyclase activity recovered in the supernatant was greater in the cortex. Taurodeoxycholate increased activity of the particulate cyclase but decreased that of the supernatant enzyme. Activity of the particulate was increased ca. 200% and that of the supernatant 500 to 700% by 1 mM NaN_3. Effects of these agents were similar in all slices. Supernatant activity was markedly increased by Co^{++} which had no effect on the particulate enzyme. In the standard assay (with 1 mM GTP, 5 mM $MnCl_2$, pH 7.4) Co^{++} activation was half-maximal with 0.02 mM and maximal with 0.1 mM. Incubation of kidney slices with 2 mM Co^{++} did not alter their cGMP content; it caused a small but significant increase in the cAMP content of slices containing medullary tissue.

Effects of assay pH on guanylate cyclase activity in fresh supernatants from all slices were similar. Basal activity increased linearly with pH from 5.9 to 9, whereas in the presence of Co^{++} there was a clear maximum at pH 7.3 to 7.5. Incubation of fresh supernatant fractions at 37° for 3 h increased guanylate cyclase activity and abolished Co^{++} activation. Cyclase from inner slices was activated somewhat more by incubation or by Co^{++} than was that from outer slices. Although the relationship between Co^{++} activation and that resulting from incubation remains to be defined, it seems probable that these phenomena reflect regulatory properties of the supernatant guanylate cyclases of kidney and other tissues.

Hepatic guanylate cyclase. Last year guanylate cyclase from guinea pig kidney was partially purified by ammonium sulfate precipitation and gel filtration. We have continued to study the properties and characteristics of guanylate cyclase, and have begun to further purify the enzyme from rat liver, a source more readily available in large quantity than guinea pig kidney.

Some of the properties of a soluble (100,000 x g supernatant) guanylate cyclase from liver are similar to those of the kidney enzyme. In particular we studied effects of Co^{++}, assay pH, and incubation at 37°C on the liver enzyme. 0.1-0.2 mM $Co(NO_3)_2$ (in the presence of 5 mM $MnCl_2$) produced maximal activation of the soluble liver enzyme; 0.04 mM produced half-maximal activation. Of the various metal ions tested, including Ca, Mg^{++}, Ni, Pb, Cu, Zn, Sr, Ba, Fe^{2+}, Fe^{3+}, none have been found to substitute for Co^{++}. Guanylate cyclase activity of fresh supernatants shows an absolute dependence on $MnCl_2$ for activity and neither Co^{++} nor Mg^{++} could substitute for $MnCl_2$ or altered the K_m for Mn^{++}. Liver guanylate cyclase was activated 10-fold by 1 mM NaN_3 and 2-3 fold by 10 or 25 mM ascorbate. The effects of Co^{++} were additive to that of NaN_3 or ascorbate. As found with the kidney enzyme, basal activity of the soluble liver enzyme increased linearly as the pH of the assay increased from 5.9 to 9, whereas in the presence of Co^{++} there was a pH maximum at about pH 7.5. As also found with the kidney enzyme, incubation of the fresh supernatant enzyme at 37° or dialysis and/or storage of 0°C increased guanylate cyclase activity and decreased the magnitude of the activation by Co^{++}. Co^{++} activated guanylate cyclase activity of fresh liver supernatant, but after elution of the soluble guanylate cyclase from DEAE Bio-Gel columns the enzyme was no longer activated by Co^{++}.

The soluble hepatic guanylate cyclase has been purified about 500-fold using ammonium sulfate fractionation, DEAE Bio-Gel ion exchange chromatography, gel filtration on Agarose A-0.5 columns, sucrose gradient (5-20%) centrifugation and preparative disc gel electrophoresis. Analytical disc gel electrophoresis of our best preparations demonstrated that guanylate cyclase activity was associated with at least 1 or 2 bands of contaminating proteins.

During DEAE Bio-Gel chromatography of the liver enzyme, material which markedly stimulates guanylate cyclase activity could be readily separated from the enzyme. This material has not yet been characterized.

Last year we described inhibitor of guanylate cyclase in fresh supernatants (100,000 x g) from guinea pig kidney. An inhibitor has also been found in fresh liver supernatants. The inhibitor from liver is a heat stable, dialyzable compound which is eluted from Sephadex G-25 columns with a K_d=0.6. The material which is eluted from the G-25 column (G-25 Inhibitor) does not inhibit guanylate cyclase activity of a fresh liver (100,000 x g) supernatant but does inhibit the activity of a dialyzed 100,000 x g supernatant or of a supernatant partially purified by Bio-Gel P-100 chromatography. The degree of inhibition was proportional to the amount of the G-25 inhibitor; maximal inhibition never exceeded 75%.

Since Co^{++} activated the fresh supernatant enzyme and not the enzyme eluted from DEAE Bio-Gel columns, and since the G-25 inhibitor affected partially purified guanylate cyclase preparations, we tested the effect of both Co^{++} and the G-25 inhibitor on the enzyme partially purified by Bio-Gel P-100 chromatography. Co^{++} was found to reverse the effect of the G-25 inhibitor. The concentration of Co^{++} required for complete reversal of the inhibition was proportional to the amount of G-25 inhibitor added. It would

appear that activation of the soluble cyclase by Co^{++} may be related to Co^{++} counteracting the effect of the heat stable inhibitor.

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 especial importance in the development, physiology, and pathology of lung, vascular smooth muscle and kidney.

Proposed Course:

We shall further purify the guanylate cyclase from rat hepatic tissue and purify and characterize the inhibitor in order to establish the mechanisms by which Co^{++} and inhibitor regulate the guanylate cyclase activity.

Publications:

1. Vaughan, M.: Metabolism of cyclic GMP in vascular smooth muscle, leukocytes, and lung. In Dumont, J. E., Brown, B. L., and Marshall, N. J. (Eds.): Eukaryotic Cell Function and Growth. Regulation by Intracellular Cyclic Nucleotides. New York, Plenum Press, 1976, pp. 369-378.

2. Clyman, R. I., Manganiello, V. C., Lovell-Smith, C. J., and Vaughan, M.: Calcium uptake by subcellular fractions of human umbilical artery. Am. J. Physiol. 231: 1074-1081, 1976.

3. Clyman, R.I., Manganiello, V.C., and Vaughan, M.: Oxygen: Influence on cyclic nucleotides and calcium sequestration in the human umbilical artery. Chest 71S: 247-249, 1977.

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6. Ting, C. C., Tsai, S.-C., and Rogers, M. J.: Host control of tumor growth. Science 197: 571-3, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 00609-09 CM

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Regulation of Hormone Sensitive Lipase Activity

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

PI:	S.-C. Tsai	Research Chemist	CM NHLBI
OTHER:	M. Vaughan	Chief, LCM	CM NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Cellular Metabolism

S:

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20014

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0.25	0.25	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Work on purification of the hormone-sensitive lipase and characterization of the lipase inactivation system has been largely suspended during the past year but will be resumed in the future.

PHS-6040

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 00610-02 CM

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Activation of Adenylate Cyclase by Hormones and Choleragen

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

PI:	Peter S. Ross	Staff Associate	CM NHLBI
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	J. Moss	Research Associate	CM NHLBI

COOPERATING UNITS (if any)
None

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NHLBI, NIH, Bethesda, Md. 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Homologous amino acid sequences in choleragen and glycopeptide hormones such as thyrotropin have led to speculation that choleragen and glycopeptide hormones activate adenylate cyclase by similar mechanisms. We have studied adenylate cyclase of a particulate preparation of bovine thyroid. Nicotinamide adenine dinucleotide (NAD) is required for activation of the enzyme by choleragen, but not by thyrotropin. Thyrotropin bound to thyroid particles and subsequently eluted retained the capacity to activate adenylate cyclase, whereas exposure to thyroid particles greatly diminished the capacity of choleragen to activate adenylate cyclase.

Project Description:

Objectives. Similarities in primary structure between choleragen and glycopeptide hormones such as thyrotropin and human chorionic gonadotropin (hCG) have led to speculation that choleragen and glycopeptide hormones activate adenylate cyclase by similar mechanisms. We have studied activation of adenylate cyclase by choleragen and thyrotropin in bovine thyroid particles.

Methods. Thyroid adenylate cyclase is prepared by suspending a 1,000 x g to 35,000 x g pellet of homogenized fresh bovine thyroid glands. Adenylate cyclase activity is measured by isolation of labeled cAMP formed by the action of thyroid particles on $\alpha[^{32}P]ATP$. NAD glycohydrolase is measured by methods previously described by this laboratory.

Major Findings. NAD was required for maximal activation of adenylate cyclase by choleragen but not by thyrotropin.

Relatively pure preparations of hCG and thyrotropin exhibited NAD glycohydrolase activity, as does choleragen. However, this enzymatic activity appeared to be a contaminant of the hCG. (Inadequate amounts of thyrotropin were available to attempt to reproduce these findings with that hormone.)

Significance to Biomedical Research:

Adenylate cyclase plays a central role in regulation of metabolic processes.

Proposed course:

Interaction of the calcium dependent heat stable activator with adenylate cyclase will be studied.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00612-01 CM

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Studies on the Mechanism of Transport of Cholesterol from Human Skin Fibroblasts

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

PI:	W. Gamble	Guest Worker	CM NHLBI
OTHER:	J. Avigan	Research Chemist	CM NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Cellular Metabolism

SI

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	1.3	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)

We have studied the mode of cholesterol efflux (reverse cholesterol transport) from human skin fibroblasts grown in a defined medium after loading with low density lipoprotein and hyperlipemic rabbit serum, by determining the intracellular cholesterol and cholesterol ester content. High density lipoprotein and delipidated serum decreased cellular cholesterol content indicating an apparent cholesterol efflux.

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Objectives and background:

Experimental evidence indicates, and it is generally agreed, that low density lipoproteins, which transport the majority of serum cholesterol, are taken up and degraded primarily by peripheral tissues. The degradation and excretion of cholesterol, however, occurs in the liver. What then, if any, is the mode of transfer of cholesterol from peripheral tissues to the liver? Reverse transport of cholesterol has been suggested as the mechanism by which cholesterol is transferred from peripheral tissues to the liver. High density lipoprotein (HDL) has been proposed as the transport agent (Miller and Miller, Lancet 16, Jan. 4, 1975; Stein et al, Biochim. Biophys. Acta 380: 106, 1975; Burns and Rothblat, Biochim. Biophys. Acta 176: 616, 1969; Bondjers and Bjorkerud, Artery 1: 3, 1974). However, the mechanism by which efflux of cholesterol from cells occurs is not known. Therefore, we have investigated the uptake and efflux of cholesterol in human fibroblasts grown in tissue culture using biochemical and morphological techniques.

The following questions were addressed:

1. What conditions contribute to an elevation of cellular cholesterol in human fibroblast grown in tissue culture?

2. What experimental conditions facilitate cholesterol egress from human fibroblasts grown in tissue culture?

3. During cholesterol efflux what are the observable morphological changes?

4. Is all of the cholesterol released in the unesterified form?

5. What, if any, is the cholesterol acceptor in the reverse cholesterol transport process and is such an acceptor an obligatory component of the transport system?

Methods:

Human fibroblast were grown to confluency according to the method of Dr. Howard Kruth of this laboratory. The cells were incubated for two days with Eagle's minimal essential medium (MEM), MEM plus hyperlipemic rabbit serum, and MEM plus low density lipoprotein (LDL). Hyperlipemic rabbit serum and LDL (human and dog) were employed to increase the cellular content of cholesterol. Subsequently the cells were treated with MEM or MEM plus HDL for two days. The cells were monitored by phase contrast and polarizing light microscopy in collaboration with Dr. Howard Kruth of this laboratory. The cells were harvested at predetermined times and free and total cholesterol determined by a method developed for the purpose (described below). Protein was determined by the method of Lowry.

Determination of free and total cholesterol. Total and free cholesterol were determined by means of an enzymatic fluorometric method developed especially for cells grown in culture. Esterified cholesterol is hydrolyzed by cholesterol esterase. Free cholesterol is oxidized by cholesterol oxidase. The peroxide formed in the latter reaction, in the presence of peroxidase, reacts with parahydroxyphenylacetic acid to form a stable fluorescent dimer. Fluorescence was measured by excitation at 325 nm and emission at 415 nm. The amount of fluorescence is directly proportional to the amount of cholesterol oxidized. Parahydroxyphenylacetic acid exhibits no fluorescence at the indicated wave lengths. One can detect as little as 0.5 µg of cholesterol. To determine free cholesterol only, cholesterol esterase is omitted from the incubation mixture. The results were comparable to those obtained with a colorimetric procedure which employs the Tschugaeff reaction and by gas chromatography. Esterified cholesterol was determined by subtracting the amount of free cholesterol from the total amount. One can manually determine cholesterol in 100 samples in about 4 h using the fluorometric method that we have described.

Findings:

Cells grown to confluency in MEM containing 10% fetal calf serum did not significantly increase their cholesterol content after treatment with hyperlipemic rabbit serum or human LDL whereas cells grown in serum-free MEM and then transferred to MEM plus hyperlipemic rabbit serum or MEM containing LDL increased their cholesterol content. Free cholesterol was increased ca. 100% (to about 100 µg/mg cell protein) and esterified cholesterol as much as 10-fold (to a level of 60-80 µg/mg cell protein). These values are comparable to those obtained by Stein et al. (Atherosclerosis 26: 405, 1977) who used chloroquine to block lysosomal metabolism of lipoproteins and cholesterol esters. Cells previously "loaded" with cholesterol by treatment with LDL or hyperlipemic rabbit serum exhibited a decrease in cholesterol content after incubation for 48 h with dog HDL_2 (64% decrease in total cholesterol) with human HDL (28-42% reduction), delipidated serum (51% reduction or MEM (ca. 28% reduction). Similar results were obtained with human HDL using much shorter periods of incubation.

It would be of interest to ascertain the amount of cholesterol that is excreted into the medium under the present experimental conditions. To that end we have attempted to determine the amount of cholesterol in the media of HDL-treated and MEM-treated "loaded" cells. Our preliminary results indicate that cholesterol is excreted into the medium. Microscopic examination of fibroblasts treated with LDL or hyperlipemic rabbit serum showed the presence of lipid inclusions in about 4 h. After 2 days some of the inclusions were found to be birefringent and exhibited the symmetrical black formee cross characteristic of inclusions (cholesterol rich) found in atherosclerotic plaques. Most of the inclusions were no longer present after the loaded cells were treated with HDL.

Significance to Biomedical Research:

Lipoproteins and cholesterol are among the primary risk factors in the development of atherosclerosis. An understanding of the process by which cholesterol is removed from peripheral cells seems essential. The use of cells grown without serum permits the study of reverse cholesterol transport in a defined system. Moreover, since there are no proteins in the medium, it should be possible to isolate and identify any acceptor molecules synthesized by the cells or those added for experimental purposes.

Proposed Course:

We propose to subfractionate the cells and isolate the inclusions and lysosomal fractions, and to follow the cholesterol-cholesterol ester content during the unloading process. We will determine the nature and amount of cholesterol excreted into the MEM medium and the HDL medium (short term).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00613-01 CM

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Role of Lysosomes in Cholesterol Uptake, Metabolism, and Release by Cultured Cells

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

PI:	Howard Kruth	Staff Associate	CM NHLBI
	M. Vaughan	Chief, LCM	CM NHLBI

COOPERATING UNITS (if any)

Laboratory of Technical Development, NHLBI
Pathology Branch, NHLBI

LAB/BRANCH

Laboratory of Cellular Metabolism

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md 20014

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)

We are investigating the formation of cholesterol-containing lipid droplets in cultured human foreskin fibroblasts exposed to human low density lipoprotein and the possible role of lysomes in this process.

PHS-6040
(Rev. 10-76)

Objectives

(1) To establish a cultured cell system in which cholesterol metabolism can be studied under controlled conditions in a chemically defined medium, (2) to correlate changes induced by low density lipoprotein in cellular cholesterol and lysomal enzyme content with changes in cellular morphology, and (3) to elucidate the mechanism of formation of lipid droplets containing cholesterol.

Methods

Normal human foreskin fibroblasts have, for the first time that we are aware of, been grown in a completely chemically defined medium (i.e., Eagle's minimal essential medium). Subcultures are initiated in minimal essential medium plus 10% fetal calf serum as usual, but 24 hr later the medium is changed and the cells are allowed to grow to confluency or near confluency in minimal essential medium without serum before incubation with human low density lipoprotein (kindly supplied by the Laboratory of Experimental Atherosclerosis and Molecular Disease Branch, NHLBI).

Cellular lysosome content is monitored biochemically by a fluorometric assay for acid phosphatase and morphologically by fluorescence microscopy (with facilities supplied by Section on Pathology, NHLBI) of living cells stained with acridine orange (which is concentrated in lysosomes). Instrumentation is being developed (with the cooperation of the Laboratory of Technical Development) to measure acid phosphatase in single cells by quantitative fluorescence microscopy using fluorogenic substrates. Cholesterol will be measured by a fluorometric assay recently adapted for use with the cultured cells by Dr. W. Gamble (Laboratory of Cellular Metabolism). Filipin, a fluorescent polyene antibiotic which binds to sterols, is used to localize cellular cholesterol by fluorescence microscopy. In addition, the techniques of phase, dark field, and polarization microscopy are employed to follow development of cellular inclusions induced by incubation of cells with low density lipoprotein. Electron microscopy and appropriate cytochemistry will also be used to investigate the mode of formation of lipid droplets that appear in fibroblasts exposed to low density lipoprotein. It is hoped that subcellular fractionation of fibroblasts can be accompanied by electron microscopy of the separated organelles.

Major Findings

The initial phase of this project has involved development of methods, but preliminary results will be noted. As mentioned above, it has been shown that normal human fibroblasts can be grown to confluency in a completely chemically defined serum-free medium. Fibroblasts grown in this serum-free medium undergo a change in morphology in which they initially lose their intracellular phase-refractile lipid inclusions and assume a flattened appearance. They remain flattened at confluency and do not form multilayers or become spindle shaped as do human fibroblasts grown in medium containing serum.

Human fibroblasts grown in serum-free medium accumulated more lipid-like inclusions on exposure to hyperlipemic rabbit serum than did cells previously grown in medium containing normal serum. The initial cholesterol content of the former cells was ca. 50 µg/mg cellular protein and this was increased to 133 µg/mg cellular protein after 48 hours with hyperlipemic serum. The fibroblasts grown in serum-free medium and subsequently incubated with low density lipoprotein accumulated many lipid inclusions during the first 24 hr of exposure. Examination by polarization microscopy between 24 and 48 hr revealed that an increasing number of inclusions demonstrated a formée (Maltese) cross (anisotropic birefringence) typical of the cholesterol ester-containing inclusions described in atherosclerotic lesions of humans.

Significance to Biomedical Research

The cells cultured as described above should provide an excellent model system in which to define the mechanisms that control cholesterol transport and metabolism and to further our understanding of the evolution of cholesterol-containing lipid inclusions which are a predominant part of the atherosclerotic lesion.

Proposed Course

To utilize this defined tissue culture system in an attempt to realize the objectives outlined above.

Annual Report of the
LABORATORY OF CHEMICAL PHARMACOLOGY
National Heart, Lung, and Blood Institute
July 1, 1976 to September 30, 1977

EFFECTS OF DRUG METABOLISM ON DRUG THERAPY AND TOXICITY

Although drug metabolism is generally considered as a mechanism of drug inactivation, many drugs are converted in the body to metabolites that evoke pharmacological and toxicological effects. Most of these biologically active metabolites are chemically inert. But during the past few years this laboratory has demonstrated that many drugs cause tissue lesions such as liver and lung necrosis through the formation of chemically reactive metabolites.

With an integrated approach consisting of both in vivo and in vitro studies, we have shown that halogenated benzenes, phenacetin, acetaminophen, isoniazid, iproniazid, and furosemide cause liver damage by chemically reactive metabolites. Because of the chemical instability of such metabolites, however, their identification has been difficult. Thus a major emphasis of the laboratory has been the identification of reactive metabolites by indirect approaches and the elucidation of the mechanisms by which the chemically reactive metabolites are formed and inactivated.

As has been repeatedly emphasized by this laboratory, the organs affected by a given chemically reactive metabolite depend not only on their intrinsic sensitivity to the toxic metabolite but also on the pharmacokinetic relationships of the parent drug and its metabolites. To show these relationships the equations for various pharmacokinetic models have been derived and the validity of the assumptions made in their derivation is being studied.

Activation of phenacetin and acetaminophen - As shown in previous studies, large doses of these commonly used analgesic drugs usually cause liver necrosis in hamsters but rarely in rats. Studies on the covalent binding of radiolabeled drugs after various pretreatments indicated that both drugs evoke their toxic effects through the formation of chemically reactive metabolites. However, phenacetin can be activated to chemically reactive metabolites by at least three different mechanisms: Mechanism 1) Phenacetin is first converted to N-hydroxyphenacetin. Although this metabolite does not rapidly react with cellular macromolecules, it can be converted to chemically reactive metabolites through the formation of either its NO-sulfate or NO-glucuronide conjugates. The NO-glucuronide conjugate has been isolated and its conversion to various breakdown products has been studied in buffer under physiological conditions. By trapping the reactive metabolite with protein or glutathione or by reducing it with ascorbic acid and studying changes in the pattern of breakdown products, we found that the formation of acetaminophen and acetamide (plus presumably quinone) occurred through the formation of the reactive metabolite, whereas phenacetin and 2-hydroxyphenacetin-O-glucuronide were formed directly from the NO-glucuronide of phenacetin. In collaboration with Dr. S. Nelson, Section on Clinical Pharmacology and Metabolism (OD:NHLBI) studies with $H_2{}^{18}O$ revealed that the oxygen in the para position of phenacetin was completely replaced by aqueous oxygen during the formation of acetamino-

phen. Thus it appears that the NO-glucuronide and probably the NO-sulfate conjugates of N-hydroxyphenacetin spontaneously decompose to N-acetylimidoquinone (the reactive metabolite), and ethanol. Another chemically reactive metabolite may preceed the formation of N-acetylimidoquinone; in the presence of phosphate buffer, a phosphorylated metabolite of phenacetin was formed at the expense of acetaminophen and acetamide. Thus several hitherto unsuspected pathways of drug metabolism have been disclosed by these studies: A) The formation of 2-hydroxyglucuronide phenacetin by an intramolecular rearrangement of the N-hydroxyglucuronide of phenacetin. B) The formation of a phosphate conjugate of phenacetin by a nonenzymatic mechanism. C) The de-ethoxylation of phenacetin.

Mechanism 2) Previous studies with Dr. Nelson revealed that liver microsomes convert phenacetin to an epoxide and then to a glutathione conjugate of acetaminophen rather than phenacetin. Studies with $^{18}O_2$ also indicated that 50% of the oxygen in the para position of the acetaminophen-glutathione conjugate was replaced by atmospheric oxygen. By contrast none of the oxygen in this position was replaced by atmospheric oxygen when the conjugate was formed directly from acetaminophen. Recent studies have revealed that the formation of the conjugate occurs through a complex series of reactions many of which were hitherto unsuspected.

Mechanism 3) Liver microsomes convert phenacetin to acetaminophen and acetaldehyde. Acetaminophen is then converted to its active metabolite. According to this mechanism no ^{18}O in ^{18}O-labeled phenacetin would be lost during the formation of the acetaminophen-glutathione conjugate. Studies with ^{18}O-labeled phenacetin administered to hamsters revealed that at least 70% of the mercapturic acid formed in the body must have arisen by the third mechanism.

Mutagenesis of N-hydroxy compounds - The concept that NO-sulfate and NO-glucuronide conjugates may be highly chemically reactive metabolites was first suggested by J.A. Miller, E.A. Miller and their associates to account for the carcinogenic effects of N-hydroxy-2-acetylaminofluorene (NOH-2AAF). In accord with this view, NO-sulfate-2AAF is much more chemically reactive than NOH-2AAF. But as was reported last year, we found in collaboration with Dr. S. Thorgeirsson, NICHHD, that mutagenesis of N-hydroxy-2AAF as measured by the Salmonella Test System of Ames is greatly enhanced by the soluble fraction of liver even in the absence of cofactors; presumably, the fraction hydrolyzes N-hydroxy-2AAF to N-hydroxy-2-aminofluorene, a known potent mutagen. We also found that addition of the cofactors required for the formation of the NO-sulfate of 2AAF decreased but did not completely prevent the mutagenic effects. Hence it still seemed possible that a part of the mutagenic effects of N-hydroxy-2AAF could still be mediated by the reactive metabolite derived from the NO-sulfate conjugate of 2AAF. However, ascorbic acid, which markedly decreases the covalent binding of the chemically reactive metabolite derived from NO-sulfate-2AAF presumably by reducing it to 2AAF, markedly increased the mutagenicity. Since neither 2AAF nor ascorbic acid are mutagenic, the increase in mutagenicity caused by ascorbic acid is probably due to the formation of a free radical of 2AAF. Whatever the mechanism of mutagenicity may be, however, it is evident that we cannot automatically assume that the most chemically reactive metabolite of a substance is necessarily the most mutagenic

metabolite. Whether the most chemically reactive metabolites are the most carcinogenic, however, remains debatable.

Activation of chloroform - In a previous study we found that in animals pretreated with phenobarbital, chloroform decreased the amount of glutathione in liver and that the rate of depletion was greater with $CHCl_3$ than with $CDCl_3$. These findings suggested the possibility that chloroform was first hydroxylated to form trichlorohydroxymethane which spontaneously decomposed to hydrochloric acid and phosgene and the phosgene reacted with glutathione. During the past year this view was confirmed by showing that rat liver microsomes converted chloroform to a metabolite which reacts with cysteine to form 2-oxothiaxolidine-4-carboxylic acid. Moreover, studies with $^{18}O_2$ revealed that the oxygen in the 2-position came from atmospheric oxygen.

Activation of chloramphenicol - Last year, we reported that a chemically reactive metabolite of chloramphenicol was formed by a reaction analogous to that which forms the reactive metabolite of chloroform; that is the dichloroacetyl group undergoes hydroxylation to form a hydroxy dichloroacetyl intermediate that spontaneously decomposes to hydrochloric acid and an oxalyl chloride intermediate. We are now attempting to identify which amino acids in protein react with the intermediate. During the past year, however, we also found that chloramphenicol is metabolized by another reaction. At 24 hr after the administration of ^{14}C-dichloroacetate or chloramphenicol labeled with ^{14}C in the dichloroacetyl group, the proteins in blood plasma are highly radioactive. But virtually all of the radioactivity is due to ^{14}C-glycine and ^{14}C-serine. Thus the dichloroacetyl group apparently undergoes hydrolytic dechlorination to form a hydroxymonochloroacetyl derivative which breaks down to form hydrochloric acid and glyoxal, a precursor in the synthesis of glycine and serine. The relative importance of the two pathways of chloramphenicol metabolism, however, remains to be determined.

Mechanisms possibly mediated by superoxide - a) Activation of estradiol. Recently other laboratories have shown that estradiol is hydroxylated to 2-hydroxyestradiol by an enzyme in brain. In collaboration with Dr. Nelson (OD:NHLBI), we have detected cytochrome P-450 in brain preparations and have found that this cytochrome P-450 is involved in the conversion of estradiol to a metabolite that becomes covalently bound to brain protein in vitro. Because the covalent binding of 2-hydroxyestradiol appears to be caused by superoxide formed from NADPH-cytochrome c reductase rather than cytochrome P-450 in brain, it seems likely that the cytochrome P-450 catalyzed the hydroxylation of estradiol.

b) Paraquat. Many laboratories have suggested that paraquat causes lung lesions by promoting the formation of superoxide. In support of this view they have shown that lipid peroxidation presumably mediated by superoxide, is increased by paraquat in the presence of microsomal preparations or purified NADPH-cytochrome c reductase. However, no one has obtained direct evidence that the steady-state concentration of superoxide in intact cells is increased sufficiently to account for the lung toxicity. During the past year, we have made another attempt, based on the oxidation of catechols by superoxide to ortho quinones which become covalently bound to tissue macromolecules. With

lung microsomes, paraquat enhances covalent binding of epinephrine but paraquat had no effect on the covalent binding of epinephrine in lung slices. It is possible, however, that the failure to detect covalently bound epinephrine may be due to glutathione present in certain types of lung cells as well as to the presence of superoxide dismutase. Our search for a better method to detect intracellular superoxide continues.

Nitrofurantoin - This drug, used in the treatment of urinary tract infections, is known to cause severe and potentially fatal lung disease in patients. In collaboration with Drs. M. Boyd (NCI) and J.R. Mitchell (OD:NHLBI) we have discovered that the toxicity may be due to an enhancement of oxygen toxicity. Feeding rats a vitamin E deficient diet containing large amounts of polyunsaturated fats greatly potentiates the toxicity of the drug. Moreover, the toxicity of the drug is also potentiated by exposing animals to a pure oxygen atmosphere. Thus the toxicity caused by nitrofurantoin in many ways mimicks the toxicity of paraquat. Unlike paraquat, however, nitrofurantoin is reduced to a chemically reactive metabolite that becomes covalently bound to tissue macromolecules. However, the covalent binding of the drug to lung macromolecules in vivo is less when the animals are in a pure oxygen atmosphere than when they are in air. Thus the data suggest that a lung enzyme reduces nitrofurantoin to a reactive metabolite which either undergoes autooxidation to form superoxide or becomes covalently bound to tissue macromolecules. As with studies on paraquat toxicity, however, nitrofurantoin did not cause detectable lipid peroxidation in vivo.

Although it has been suggested that superoxide formation might mediate the mutagenic effects of nitrofurans, including nitrofurantoin, we have found no evidence to support this view. Indeed, nitrofurantoin causes more frameshift mutations under anaerobic conditions than in air in the Salmonella Test Systems. Moreover superoxide generated by xanthine oxidase does not cause mutations. Thus nitrofurantoin appears to cause cellular damage and mutagenic changes by different mechanisms.

Effects of an active metabolite of spironolactone - Several years ago we reported that high doses (100 mg/kg) of spironolactone caused a decrease in testicular cytochrome P-450 in all animal species and a decrease in adrenal cytochrome P-450 in cortisol producing animals without causing cellular damage and that the decrease in the cytochrome P-450 was reversible on withdrawal of the drug. Accordingly there was a decrease in 17α-hydroxylase activity in the affected organs. In recent experiments, spironolactone also decreased the activity of 17α-hydroxylase in rat ovaries and the activity of C_{17}-C_{20} lyase, another enzyme required in the synthesis of 17-keto steroids, in testis and adrenals. The destruction of cytochrome P-450 caused by spironolactone apparently parallels the 17α-hydroxylase in cells. Thus, it occurs in Leydig cells in testis of all species and preferentially in cells located in the zona fasciculata of adrenals in cortisol producing animals. The mechanism by which spironolactone causes the decreases in cytochrome P-450 is not completely understood. Previous studies revealed that a decrease in heme occurs in testis and adrenals. It now appears that apoproteins of cytochrome P-450 as well as heme are lost in animals. In vitro experiments have revealed that the spironolactone-induced destruction of cytochrome P-450

requires NADPH and oxygen and is inhibited by progesterone, indicating that the destruction probably is mediated through an active metabolite. Because the destruction cannot be prevented by glutathione, however, the active metabolite may not be chemically reactive. Structure-activity studies reveal that the thio group in the 7α-position of spironolactone is essential for cytochrome P-450 destruction. Thus the 7α-thio analogue of spironolactone is more potent than spironolactone in evoking the effect. But SC 25152 (which has a $O\text{-}CO\text{-}CH_3$ group instead of a $S\text{-}CO\text{-}CH_3$ group in the 7α-position) and aldadiene do not cause cytochrome P-450 destruction.

It now seems likely that the estrogen-like side effects of spironolactone are mainly due to an interaction of spironolactone with testosterone receptors rather than to a decrease in testosterone synthesis. Loriaux (NICHHD),in collaboration with us, has found that SC 25152 not only does not destroy cytochrome P-450 but that it has a lower affinity than spironolactone for α-dihydrotestosterone receptors. Thus, SC 25152 may cause fewer estrogen-like side effects than does spironolactone.

1-Chloro-2-[2,2-dichloro-1-(4-chlorophenyl)ethyl]benzene (o,p'-DDD; Mitotane) - This adrenal cytotoxic agent has been used in the treatment of inoperable adrenal cortical carcinoma. But the mechanism of the cytotoxicity is not clearly understood. During the past year it was found that o,p'-DDD in dogs caused not only adrenal atrophy and necrosis but also decreases in cytochrome P-450 in adrenal mitochondria and microsomes. Prior treatment of the animals with spironolactone blocked the further decrease in adrenal cytochrome P-450 and prevented the cytotoxicity. Thus, it seems possible that the cytotoxic action of o,p'-DDD may be due to an active metabolite formed from o,p'-DDD by an adrenal cytochrome P-450 enzyme. It is noteworthy, however, that o,p'-DDD does not cause frameshift mutations in the presence of microsomes from dog or human adrenals or from rat liver.

Pharmacokinetics - In the past, simple pharmacokinetic models have been invaluable in reconciling seemingly discordant data obtained in our drug toxicology studies. However, the equations for models in which drugs and their metabolites are rapidly eliminated simultaneously by several organs are much more complex. Indeed many of the assumptions made in the derivation of the pharmacokinetic equations for systems limited by organ blood flow may not be valid. During the past year we have devised a method for evaluating the validity of many of the assumptions by studying the metabolism of phenacetin and its metabolite, acetaminophen, in liver perfusion systems. Studies of acetaminophen metabolism in rats have revealed that the apparent intrinsic clearance ($Cl_{int} = V_m/K_m$) of the PAPS transferase *in vivo* is virtually identical to that found in a liver perfusion system. These data indicate the validity of using measurements of the areas under the blood concentration curves after intraperitoneal and intravenous administration of acetaminophen for estimating the extraction ratio of the drug as it passes through the liver and the liver blood flow rate in rats. Our calculations indicated, however, that the blood flow rate was about 25-30 ml/min in 350 g rats, a rate which is considerably higher than the 10 ml/min generally believed to occur. Nevertheless, the finding that the extraction ratio $[p\,Cl_{int}/(p\,Cl_{int} + Q)]$ of acetaminophen formed from phenacetin within hepatocytes is smaller than the

extraction ratio of preformed acetaminophen has shown for the first time that intercellular differences in the distribution of cytochrome P-450 and sulfuryl transferase can be functionally important in flow limited systems. These findings are especially important for drugs that evoke their effects through the formation of an active metabolite.

Potentiation of drug toxicity by isoproterenol - Increases in the intensity and duration of action of drugs caused by a substance that slows their elimination from the body are usually due to inhibition of the metabolism of the drugs. But last year we reported that high doses of isoproterenol (0.3 mg/kg, s.c.) potentiated the toxicity of paraquat by inhibiting its excretion by the kidney and that this effect could be blocked by propranolol. We recently found that isoproterenol also potentiates the toxicity of tetraethylammonium bromide, decamethonium and hexamethonium, which are also rapidly excreted into urine. The finding that isoproterenol decreases the clearance of inulin as well as para aminohippuric acid and N-methylnicotinamide suggests that isoproterenol causes reversible impairment of kidney function by a mechanism that remains obscure.

PHYSIOLOGICAL CONTROL MECHANISMS

Relationships between cyclic AMP (cAMP), cyclic GMP (cGMP) and Ca^{++} - Various hormones are known to mobilize Ca^{++} from tissue stores, increase the tissue concentrations of cAMP and cGMP and evoke various physiological responses in different tissues. However, the relationships between these changes are incompletely understood. An objective of this laboratory has been to elucidate which changes are associated with a given physiological response and the sequence of events which lead to the response.

Pancreas - Various secretagogues including carbamylcholine and caerulin increase the efflux of Ca^{++}, the concentration of cGMP and the rate of amylase secretion. But the secretagogues do not increase the concentration of cAMP. Moreover, calcium, but not the secretagogues, increases the activity of pancreatic guanylate cyclase in cell-free preparations; thus the secretagogues appear to act by mobilizing calcium and calcium in turn activates guanylate cyclase. Although the rate of amylase secretion may be doubled either by the calcium ionophore, A23187, or by dibutyryl cGMP, this increase is considerably less than that obtained with the secretagogues. Thus the requirements for optimal amylase secretion remain to be determined.

Pineal gland - It is well known that various beta adrenergic agonists increase both the concentration of cAMP and the activity of N-acetyl transferase (NACT) in the pineal gland. However, much of the increase in NACT activity occurs while the concentration of cAMP is falling. In collaboration with D.C. Klein, NICHHD, we have found that after the NACT reaches its maximal activity the addition of propranolol, a beta adrenergic blocking drug, causes a rapid decline in both the remaining cAMP and the NACT activity. These decreases may be reversed by dibutyryl cAMP or a phosphodiesterase inhibitor. Thus the slow increase in NACT activity may be associated with the induced synthesis of the enzyme rather than a slow activation. Indeed NACT is apparently rapidly activated and maintained in its active form by relatively small concentrations of cAMP. Moreover, cGMP may play a role in pineal neurons.

SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00803-08-LCP

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Cytochrome P-450 and NADPH Cytochrome c Reductase in Rat Brain

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

PI:	H.A. Sasame	Chemist	LCP	NHLBI
	S.D. Nelson	Staff Fellow	OD	NHLBI
OTHER:	M.M. Ames	Staff Fellow	HE	NHLBI

COOPERATING UNITS (if any)
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LAB/BRANCH
Laboratory of Chemical Pharmacology

SE
Enzyme-Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	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)

Microsomes isolated from whole rat brain were found to contain both cytochrome P-450 (0.025-0.051 nmoles/mg) and NADPH cytochrome c reductase activity (26-55 nmoles/mg/min). The brain cytochrome P-450 apparently catalyzes the oxidation of estradiol to a reactive metabolite that became covalently bound to rat brain microsomal protein. The covalent binding of estradiol was inhibited 63% by an atmosphere of $CO:O_2$. Moreover, an antibody prepared against NADPH cytochrome c reductase inhibited not only the covalent binding of estradiol but also the covalent binding of 2-hydroxyestradiol and catecholamines by rat brain microsomes.

PHS-6040

Project Description:

Objectives: Since brain is an end target organ for the action of many drugs and hormones, it seems important to assess whether brain itself possess es cytochrome P-450 enzyme systems, which are known to convert many chemicals to reactive intermediates. The reactive intermediate may react with either neurons or other vital components in brain to cause abnormal effects.

Methods Employed: Preparation of rat brain microsomes: Whole rat brain was homogenized with three volumes of nitrogen-deaerated 1.15 M KCl - 0.02 Tris buffer containing 0.5 mM dithioethitol, 0.2 mM EDTA and 15% volume of glycerol. The homogenates were centrifuged at 19,000 x g for 20 min in a Serval RC 2 centrifuge. Brain microsomes were then isolated by the same procedure used to isolate liver microsomes. Both carbon monoxide differential spectrum and estradiol induced differential spectrum of rat brain microsomes were carried out in Aminco DW 2 spectrophotometer. The methods for assaying the covalent binding of estrogens and catecholamines were similar to those which were already reported in preceeding annual reports.

Major Findings: 1) Microsomes isolated from whole rat brain contained cytochrome P-450 (0.02-0.051 nmoles/mg) and NADPH cytochrome c reductase activity (26-55 nmoles/mg/min). 2) The oxidation of estrogen to a reactive metabolite that became covalently bound to rat brain microsomes was inhibited by either $CO:O_2$ atmosphere or antibody against NADPH cytochrome c reductase indicating that the activation step for the formation of a reactive metabolite is indeed mediated by cytochrome P-450 oxygenase. On the other hand, the covalnt binding of catecholamines to microsomal protein was inhibited only by the antibody against NADPH cytochrome c reductase. 3) The addition of a substrate, estradiol to rat brain microsomal suspension induced a differential spectrum, a reverse type I, having a rough at 380 nm and a peak at 420 nm, suggesting that there is an interaction between estradiol and the heme region of cytochrome P-450.

Significance to Biomedical Research and the Program of the Institute: Because estrogens can be activated by extrahepatic tissues such as brain to metabolites that alkylate various components in tissues, studies should be focused on extrahepatic metabolism and toxicity of drugs.

Proposed Course of Project: Further search for a possible existence of cytochrome P-450 enzyme systems in extrahepatic tissues will be continued.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00804-02 LCP

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Studies on the Detoxification of Reactive Metabolites

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

PI:	J.A. Hinson	Staff Fellow	LCP	NHLBI
	H. Borner	Visiting Fellow	LCP	NHLBI
	G.J. Mulder	Guest Worker	LCP	NHLBI
	J.R. Gillette	Chief, Lab. of Chemical Pharm.	LCP	NHLBI

COOPERATING UNITS (if any)
Dr. Mulder is a Guest Worker from the State University of Groningen, The Netherlands.

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme-Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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)

The effect of glutathione (GSH), GSH transferase, and ascorbic acid on the detoxification of reactive metabolites has been studied. A simple in vitro assay has been devised to study the importance of GSH and GSH transferase on the detoxification of the microsomally generated reactive metabolites of acetaminophen, bromobenzene and naphthalene. These results show that the reactive metabolites of acetaminophen, bromobenzene and naphthalene are more efficiently detoxified in the presence of GSH and GSH transferase than by GSH alone. These data also show that the majority of the reactive epoxide metabolites of bromobenzene and naphthalene are in equilibrium with the aqueous media. These data suggest that the cytochrome P-450 and epoxide hydratase are not a coupled system. Ascorbic acid efficiently detoxified the reactive metabolite of acetaminophen in microsomal incubation mixtures but at doses of 1 gm/kg did not alter the hepatoxicity of acetaminophen. It is concluded that ascorbic acid is not an important detoxification mechanism with acetaminophen.

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Project Description:

Objectives: Previous studies in this laboratory have shown that the hepatic necrosis observed with acetaminophen, furosemide, bromobenzene and isoniazid have resulted from the formation of electrophilic reactive metabolites. Reactive metabolites have also been implicated in other toxicities such as lung edema, nephotoxicity and carcinogenesis. We have been interested in mechanisms of detoxification of various electrophilic metabolites. The role of GSH and GSH transferase in detoxification of various metabolites has been examined. A potential role for ascorbic acid detoxification has also been studies.

Methods Employed: Reactive metabolites were generated enzymatically in microsomal incubation mixtures using microsomes isolated from mouse liver. Sephadex G-25 chromatographed mouse liver supernatant was used as a source of GSH transferase. Boiled supernatant was added to the microsomal incubation mixture to study nonenzymatic detoxification of GSH. Metabolites were assayed by thin layer chromatography. All other methods were standard procedures.

Major Findings: Addition of varying concentrations of GSH to incubation mixtures containing mouse liver microsomes, ^{3}H-acetaminophen and cofactors for generation of the reactive metabolite, showed covalent binding of isotopically labeled material to macromolecules decreased to nearly zero at 0.5 mM GSH. In the presence of GSH transferase to the incubation mixture, however, covalent binding was decreased by a similar amount at 0.05 mM GSH. There was a corresponding increase in the formation of an acetaminophen-glutathione conjugate. A similar role for GSH transferase has been established with the reactive metabolites of bromobenzene and naphthalene. In the absence of added GSH the metabolism of bromobenzene was similar whether GSH transferase was present or absent. In these incubation mixtures covalent binding was a major metabolite, accounting for approximately 20% of the total metabolism while the phenols accounted for approximately 50% and the dihydrodiol accounted for 20%. In the presence of GSH (1 mM) and the absence of GSH transferase the phenolic metabolites were 50% and the dihydrodiol was 20% of the total metabolism. Covalent binding decreased to less than 10% and the GSH conjugate accounted for approximately 20% of the total metabolites. In the presence of GSH (1 mM) and GSH transferase, however, the GSH conjugate was the principal metabolite accounting for 63%, covalent binding was negligible, the phenolic metabolites were approximately 30%, and the dihydrodiol decreased to approximately 6%. Naphthalene showed a similar pattern for metabolism in the presence and absence of GSH and GSH transferase. With naphthalene, however, covalent binding accounted for less of the total metabolism than with bromobenzene.

In other experiments we showed that in vitro covalent binding of the reactive metabolite of acetaminophen was readily blocked by small concentrations of ascorbic acid indicating that the reactive metabolite is readily reducible. Since similar concentrations of GSH blocked the in vitro

covalent binding of acetaminophen and GSH is known to be important in the in vivo detoxification of the reactive metabolite, we examined the possible importance of ascorbic acid in the in vivo detoxification of the reactive metabolite of acetaminophen. Injection of ascorbic acid (1 gm/kg) to mice at the same time as an hepatoxic dose of acetaminophen did not change the toxicity of acetaminophen. Also injection of ascrobic acid 1 hr after an hepatotoxic dose of acetaminophen did not alter the toxicity of acetaminophen. These data indicate ascorbic acid is not important in the in vivo detoxification of the reactive metabolite of acetaminophen.

Significance to Biomedical Research and the Program of the Institute: The present studies show the importance of GSH transferase in the detoxification of the reactive metabolites of acetaminophen, bromobenzene and naphthalene. The data also show that the majority of the reactive epoxide metabolites of bromobenzene and naphthalene are in equilibrium with the aqueous media and the cytochrome P-450 is not coupled with the epoxide hydratase as has been previously suggested. It has also been shown that ascorbic acid which detoxifies the reactive metabolite of acetaminophen in vitro is not important in the in vivo detoxification of the reactive metabolite of acetaminophen.

Proposed Course of Project: The importance of GSH, GSH transferase and ascorbic acid in detoxification of reactive metabolites will continue to be examined.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01HL 00806-02 LCP

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Activation of N-Hydroxy-N-arylacetamides by Sulfation and Glucuronidation

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

PI:	J.A. Hinson	Staff Fellow	LCP	NHLBI
	G.J. Mulder	Guest Worker	LCP	NHLBI
OTHERS:	L.S. Andrews	Staff Fellow	LCP	NHLBI
	J.R. Gillette	Chief, Lab. of Chem. Pharm.	LCP	NHLBI

COOPERATING UNITS (if any)
Dr. G.J. Mulder is a Guest Worker from the State University of Groningen, The Netherlands. Dr. L.S. Andrews is a Staff Fellow in the Pharmacology-Toxicology Program, NIGMS.

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme-Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	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)

N-Hydroxylation is believed to be the common intermediate in the nephrotoxicity, hepatotoxicity, hemolysis, methemoglobinemia and carcinogenesis of N-aryl-acetamides. The hepatotoxicity of acetaminophen is believed to be mediated by a toxic metabolite produced by N-hydroxylation. N-Hydroxylation of polycyclic N-arylacetamides followed by N-O-sulfation is believed to produce one of the ultimate carcinogenic metabolites of these compounds. Since we have shown that the analgesic phenacetin and related compounds are N-hydroxylated we have examined the capacity of these compounds to be sulfated and glucuronidated. These N-hydroxy compounds were shown to be readily sulfated and glucuronidated. Following sulfation of N-hydroxyphenacetin or N-hydroxy-2-acetylaminofluorene a relative metabolite was immediately formed and covalently bound to protein. Following glucuronidation of N-hydroxyphenacetin a reactive metabolite was formed, but at a slower rate, the one half life was 8.7 hours. An examination of breakdown products of purified N-hydroxyphenacetin glucuronide under varying conditions implicated acetylimidoqunone as the reactive metabolite. This metabolite could either covalently bind to protein, be reduced to acetaminophen or undergo hydrolysis to quinone and acetamide.

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Project Description:

Objectives: The mechanisms of nephrotoxicity, hepatotoxicity, hemolysis, and methemoglobinemia of monocyclic N-arylacetamide are unknown. In previous studies we have shown that monocyclic N-arylacetamides are N-hydroxylated by a microsomal cytochrome P-450 mixed-function oxidase. Since the N-O-sulfate ester of polycyclic N-hydroxy-N-arylacetamide is believed to be the toxic metabolite of this carcinogen, we have studied the sulfation and glucuronidation of monocyclic ^{14}C-N-hydroxy-N-arylacetamides.

Methods Employed: ^{14}C-N-Hydroxy-N-arylacetamides were synthesized following previously described procedures. The sulfotransferase enzyme necessary for sulfation was prepared by chromatography of 100,000 x g supernatant on a Sephadex G-25 column. The glucuronyl transfeerase enzyme was prepared by activating microsomes with Triton X-100. Metabolites were isolated by thin layer chromatography and quantitated by liquid scintillation spectrometry. All other methods were standard procedures.

Major Findings: An assay has been developed for quantitating sulfation of N-hydroxy derivatives. A comparison of the rate of sulfation and glucuronidation of polycyclic N-hydroxy-N-arylacetamides to monocyclic N-hydroxy-N-arylacetamides showed they were metabolized at similar rates. With the monocyclic N-hydroxy-N-arylacetamides only N-hydroxyphenacetin, however, was converted to a reactive electrophilic metabolite which covalently bound to protein after sulfation or glucuronidation. Various nucleophiles inhibited the covalent binding to protein of the monocyclic N-hydroxy-N-arylacetamide N-hydroxyphenacetin and the polycyclic N-hydroxy-N-arylacetamide, N-hydroxy-2-acetylaminofluorene. Cysteine and glutathione but not methionine inhibited the covalent binding of the sulfate and glucuronide esters of N-hydroxyphenacetin, whereas methionine but not glutathione inhibited the covalent binding of the sulfate ester of N-hydroxy-2-acetylaminofluorene. The purine nucleosides guanosine and adenosine inhibited the covalent binding of the sulfate ester of N-hydroxy-2-acetylaminofluorene to protein but did not affect the covalent binding of the sulfate ester of N-hydroxyphenacetin to protein. Ascorbic acid inhibited the covalent binding of both N-O-sulfate esters of N-hydroxyphenacetin and N-hydroxy-2-acetylaminofluorene.

An examination of the rate of conversion of the N-O-sulfate and N-O-glucuronide esters of N-hydroxyphenacetin to a reactive metabolite showed the sulfate ester immediately rearranged to form a reactive metabolite, whereas the N-O-glucuronide slowly rearranged to a reactive metabolite. The N-O-glucuronide of N-hydroxyphenacetin from a microsomal incubation was isolated and purified. Using this purified compound the conversion to a reactive metabolite was studied. The one-half life of this compound was 8.7 hr and the following metabolites were isolated: 2-Hydroxyphenacetin glucuronide, phenacetin, acetaminophen, and acetamide. In the presence of protein covalent binding occurred and in the presence of glutathione a conjugate was formed. Isolation and identification of this glutathione conjugate showed it to be a glutathione-acetaminophen conjugate. Assays of these metabolites under varying conditions showed N-hydroxyphenacetin

glucuronide could rearrange to 2-hydroxyphenacetin glucuronide or phenacetin or decompose to a reactive metabolite, presumably acetylimidoquinone. Acetylimidoquinone could be reduced to acetaminophen, covalently bound to protein, undergo hydrolysis, hydrolyze to acetamide and quinone or combine with glutathione.

Significance to Biomedical Research and to the Program of the Institute: The present studies show that monocyclic N-hydroxy-N-arylacetamide are readily sulfated and glucuronidated. Only N-hydroxyphenacetin, however, formed a reactive metabolite that covalently bound to protein after sulfation or glucuronidation. The reactive metabolite formed after sulfation of the carcinogen of N-hydroxy-2-acetylaminofluorene was capable of reacting with purine bases of DNA, whereas the reactive metabolite formed after sulfation of N-hydroxyphenacetin was not. Acetylimidoquinone has been shown to be the reactive metabolite produced after sulfation or glucuronidation of N-hydroxyphenacetin.

Proposed Course of Project: Since glucuronidation and sulfation of N-hydroxyphenacetin leads to the formation of the reactive metabolite acetylimidoquinone, the importance of these pathways of metabolism in the toxicities of phenacetin will be examined.

Publications:

Mulder, G.J., Hinson, J.A. and Gillette, J.R.: Generation of reactive metabolites of N-hydroxyphenacetin by glucuronidation and sulfation. Biochemical Pharmacology 26: 189-196, 1977.

Mulder, G.J., Hinson, J.A. and Gillette, J.R.: Conversion of the N-O-glucuronide and N-O-sulfate conjugates of N-hydroxyphenacetin to reactive metabolites. Biochemical Pharmacology, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00807-01 LCP

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Mutagenicity of N-Hydroxy-acetylarylamides

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

PI:	L.S. Andrews	Staff Fellow	LCP	NHLBI
OTHERS:	J.A. Hinson	Staff Fellow	LCP	NHLBI
	J.R. Gillette	Chief, Lab. of Chemical Pharm.	LCP	NHLBI

COOPERATING UNITS (if any)
Dr. L.S. Andrews is a Staff Fellow in the Pharmacology-Toxicology Program, NIGMS.

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Laboratory of Chemical Pharmacology

SECTION
Enzyme-Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	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)

N-Hydroxy-2-acetylaminonaphthalene (NOH-2AAN) and N-hydroxy-2-acetylaminofluorene (NOH-2AAF) but not N-hydroxyphenacetin were activated by rat liver 100,000 x g supernatant enzymes to potent mutagens in the Ames-Salmonella mutagenicity test system. An amidase, which can be partially inhibited by paraoxon, converted NOH-2AAF to N-hydroxy-2-aminofluorene (NOH-2AF), a potent frameshift mutagen. Upon addition of sulfation cofactors, a cytosol sulfotransferase catalyzed the formation of the N-O-sulfate ester of NOH-2AAF, which is a less potent mutagen than is NOH-2AF. Ascorbic acid as well as reduced pyridine nucleotides increased the loss of acetate from NOH-2AAF and proportionally increased the observed mutagenic response. Ascorbic acid while not affecting the generation of the N-O-sulfate ester of NOH-2AAF decreased the observed protein covalent binding and increased the reduction of the sulfate ester to 2AAF. Under these conditions ascorbic acid increased the mutagenic response ten-fold while generating a nonmutagenic species, 2AAF. These results are consistent with the proposal that a radical cation intermediate generated by ascorbate is a potent mutagenic species.

Project Description:

Objectives: N-Hydroxy-acetylarylamides represent an important class of compounds to which workers are occupationally exposed as well as possible metabolites of several drugs. Several of this class of compounds have been shown to be carcinogenic in experimental animals and are also detected as mutagens in the Ames-Salmonella mammalian enzyme mutagenicity test system. This bacterial test system has recently been reported to detect most of the known mammalian carcinogens as mutagens. As such, this mutagenesis test system may represent an important advance in testing for carcinogenic and mutagenic activity. N-Hydroxy-2-acetylaminofluorene was utilized as a model compound to characterize what type of compounds are detected in this mutagenesis test system.

Methods Employed: Mutagenesis assays measured a back mutation from a requirement for supplemented histidine to prototrophy. The enzymes used in the activation of NOH-2AAF to mutagenic species were present in postmicrosomal supernatant fraction subjected to Sephadex G-25 chromatography. Correlative radiolabel studies employed (^{14}C-acetyl)-NOH-2AAF in order to elucidate the biotransformation of this compound under conditions of the mutagenesis assay. Products were identified by thin layer chromatography and subsequent reverse isotope dilution.

Major Findings: NOH-2AAF is activated to a potent mutagen by an amidase present in the rat liver supernatant fraction. Paraoxon partially inhibited the amidase as evidenced by a decreased release of ^{14}C-acetate from (^{14}C-acetyl) NOH-2AAF and proportionately decreased the observed mutagenic response. Ascorbic acid increased acetate loss from NOH-2AAF and also increased the mutagenic response. The mutagen formed in this system appears to be NOH-2AF.

Upon addition of sulfation cofactors a sulfotransferase present in the supernatant fraction converts NOH-2AAF to the N-O-sulfate ester. This N-O-sulfate ester covalently bound extensively to protein but was only weakly mutagenic. Ascorbic acid inhibited the covalent binding of the N-O-sulfate ester of NOH-2AAF to protein without affecting the rate of N-O-sulfate generation. While decreasing covalent binding to protein, however, ascorbate increased the mutagenicity by a factor of ten-fold and reduced the N-O-sulfate to 2AAF, a compound which was not mutagenic in the system used. Thus, it appears that an intermediate in the reduction of the N-O-sulfate ester of NOH-2AAF to 2AAF is a potent mutagen. Since ascorbate is known to undergo one electron oxidation-reduction, a free radical intermediate may be the mutagenic species involved. Electron spin resonance spectroscopy, however, has failed to detect this proposed free radical species indicating the radical cation may be an extremely short-lived intermediate under the conditions of this assay.

Significance to Biomedical Research and the Program of the Institute: With the increasing awareness that chemicals present in our environment may produce mutagenic or carcinogenic effects in humans, the search for a simple, rapid test system for chemical carcinogens has been intensified. The Ames-Salmonella test system is a simple rapid assay which has shown a 90% correlation between known human carcinogens and detected mutagens. This test system is of obvious importance in drug screening programs as well as testing of compounds in the environment. The importance of a negative response in such a test system must be assessed, and in order to make this assessment the potency of different types of compounds in the system should be determined. This research has thus far suggested that potent arylating species are only weakly mutagenic and that free radicals may be more important in mutagenicity and carcinogenicity than previously believed.

Proposed Course of Project: The mutagenesis of acetylarylamides and related compounds will be investigated with particular regard to interactions with the Salmonella bacterial genome. The possible mutagenicity of other free radicals will be investigated with this assay system.

Publication:

Mulder, G.J., Hinson, J.A., Nelson, W.L. and Thorgeirsson, S.S.: The role of sulfotransferase from rat liver in the mutagenicity of N-hydroxy-2-acetylaminofluorene in Salmonella Typhimurium. Biochem. Pharmac. 26: 1358-1360, 1977.

PROJECT NUMBER (Do NOT use this space) | HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | Z01 HL 00808-01 LCP

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Mutagenicity of Nitrofurantoin and Nitrofurazone

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

PI:	L.S. Andrews	Staff Fellow	LCP	NHLBI
OTHERS	R.H. Menard	Expert	LCP	NHLBI
	J.R. Gillette	Chief, Lab. of Chemical Pharm.	LCP	NHLBI

COOPERATING UNITS (if any)

Dr. L.S. Andrews is a Staff Fellow in the Pharmacology-Toxicology Program, NIGMS.

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Enzyme-Drug Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	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)

Nitrofurantoin and nitrofurazone, drugs used in the treatment of anaerobic bacterial infections, were detected as mutagens in the Ames Salmonella mammalian enzyme mutagenicity test system. Bacterial nitroreductases are important in the mutagenic activation of these drugs since no mutagenic response was obtained from bacteria deficient in nitroreductase activity. The addition of rat liver 9,000 x g supernatant to nitroreductase deficient bacteria under anaerobic conditions however produced mutations. Thus, under anaerobic conditions mammalian liver enzymes convert these two drugs to mutagenic compounds. Guinea pig testicular microsomes, unlike rat liver, could not activate these drugs to mutagens.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Many nitrofuran derivatives are currently being used in therapeutics to treat anaerobic bacterial as well as protozoal infections. These drugs are bacteriocidal by virtue of their ability to modify the bacterial genome following nitroreduction. The recent development of a mutagenesis test system which detects mammalian carcinogens has facilitated the study of these drugs as mutagens and possible carcinogens in man.

Methods Employed: Mutagenesis assays measure the reversion of a apecially constructed Salmonella strain with or without nitroreductase enzymic activity from histidine heterotrophy to prototrophy. The method thus utilizes a back mutation and the number of colonies obtained at the end of the assay reflects the degree of mutagenesis.

Tissue fractions from rat liver and guinea pig testes were prepared under sterile conditions and added with nitrofurantoin or nitrofurazone to bacterial testes strains TA 100 and TA 100 FR. After incubation for 30 min, the mixture was plated out on agar and the number of revertants measured.

Major Findings: Nitrofurantoin and nitrofurazone mutated the Salmonella strain possessing nitroreductase activity (TA 100) but did not mutate the strain deficient in nitroreductase (TA 100 FR) without the prior addition of mammalian enzymes under anaerobic conditions. Rat liver 9,000 x g supernatant enzymes effectively activated these drugs to mutagens, but guinea pig testicular microsomes did not activate the nitrofurans to mutagens.

Significance to Biomedical Research and Program of the Institute: Nitrofurans represent an important class of chemotherapeutic agents which are in extensive use. While these compounds produce their therapeutic effect by altering the genome of the infectious agent, the effects of nitrofurans on most mammalian cells remain undetermined. The ability of mammalian enzymes to substitute for bacterial nitroreductases in the activation of nitrofurans to mutagens has suggested that these compounds may also produce a mutagenic effect on mammalian DNA.

Proposed Course of Project: The ability of differential tissue fractions from mammalian germ cell tissues as well as mammalian liver to activate nitrofurans to mutagens will be determined. The individual mammalian enzymes involved in mutagenesis and the specific effects of nitrofurans on the bacterial genome will be investigated.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00809-01 LCP

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Testicular Tissue Mediated Mutagenesis

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

PI:	L.S. Andrews	Staff Fellow	LCP	NHLBI
OTHERS:	R.Menard	Expert	LCP	NHLBI
	J.R. Gillette	Chief, Lab. of Chemical Pharm.	LCP	NHLBI

COOPERATING UNITS (if any)

Dr. L.S. Andrews is a Staff Fellow in the Pharmacology-Toxicology Program, NIGMS.

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Enzyme-Drug Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

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)

2-Acetylaminofluorene and benzo[a] pyrene were activated to frameshift mutagens in the Ames-Salmonella test system (TA 1538) by rat liver tissue microsomes but not by dog testicular tissue microsomes. Both tissues possess cytochrome P-450, although testicular tissue has a much smaller amount than does liver. Testicular microsomes did not metabolize benzo [a] pyrene to any great extent and did not activate this compound to a frameshift mutagen. Mitotane, o,pDD was not activated by dog adrenal microsomes to a frameshift mutagen.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Many chemicals which are carcinogenic or mutagenic in man require metabolic activation to a reactive intermediate which reacts with the cell genome. The enzymes which mediate the metabolism of many carcinogens are present in several tissues, but the greatest activity is found in the liver. For a chemical to exert an inherited mutagenic effect in man, it must interact with the testicular genome. As many postulated ultimate chemical carcinogens are quite reactive chemically, it is questionable whether they may be produced in the liver and reach the testes unchanged. Thus, testicular mediated metabolic activation of chemicals may be of greater importance to mutagenesis than is liver metabolic activation due to instability of the reactive form. Accordingly, using the Ames-Salmonella mutagenicity test system we are investigating the ability of testicular tissue to activate known chemical carcinogens to mutagens.

Methods Employed: Mutagenesis assays measure the reversion of a specially constructed Salmonella strain from histidine heterotrophy to prototrophy. The method thus utilizes a back mutation and the number of colonies obtained at the end of the assay will reflect the degree of mutagensis. Standard microbiological techniques are employed.

Microsomes from guinea pig adrenals; dog testes and rat liver were prepared under sterile conditions and added with benzo [a] pyrene or 2-acetylaminofluorene to the bacterial tester strain TA 1538. After incubation the mixture was plated out on agar and the number of revertants measured. Cytochrome P-450 content and benzo[a] pyrene metabolic activity were measured by spectrophotometric and fluorometric assays, respectively.

Major Findings: While rat liver microsomes activated 2-acetylaminofluorene and benzo[a] pyrene to frameshift mutagens, dog testicular microsomes did not. Correlative experiments indicated that dog testes could not metabolize benzo[a] pyrene to hydroxylated metabolites which have been implicated in the mutagenesis following liver metabolic activation of this compound.

Mitotane (o,pDD), a drug currently in use in treating adrenal tumors which causes considerable damage to adrenal DNA, was not activated by either rat liver microsomes or dog adrenal microsomes to a frameshift mutagen in tester strain TA 1538.

Significance to Biomedical Research and the Program of the Institute: While the importance of detecting chemicals which are carcinogenic to man has been recently underscored, the ability of chemicals to mutate mammalian germ cells is of much greater importance to man as a species. With the realization that many anticancer drugs as well as bacteriostatic agents are mutagenic in bacterial test systems has come the importance of testing these compounds in mammals. The Ames-Salmonella mutagenesis test system which combines the use of mammalian tissues for metabolic activation and a bacterial

indicator organism affords the opportunity of determining effects of mammalian metabolites of chemicals on DNA. Our results thus far indicate that testicular tissue does not have the capability of converting 2-acetyl-aminofluorene or benzo[a] pyrene to bacterial frameshift mutagens. Other compounds, in particular nitrofuran anti-bacterial agents are currently being tested in both frameshift as well as point mutation strains.

Proposed Course of Project: The ability of nitrofuran antibacterial agents to be converted to mutagens by mammalian testicular tissue will be evaluated.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00810-01 LCP

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Pharmacokinetics of Acetaminophen Formation and Elimination in Perfused Rat Liver

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

PI:	K.S. Pang	Visiting Fellow	LCP	NHLBI
	J.R. Gillette	Chief, Lab. of Chem. Pharm.	LCP	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme-Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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)

A model of hepatic drug clearance is reviewed theooretically to describe the kinetics of the parent drug and its metabolite. The model was tested with phenacetin, and its O-deethylated metabolite, acetaminophen in the perfused rat liver in situ preparation.

211

Project Description:

Objectives: The role of pharmacologically toxic and active metabolite in therapeutics is well recognized. While there is copious pharmacokinetic data in the literature on the parent drug, very few studies on the pharmacokinetics of drug metabolites have been published. A model describing the hepatic clearance of drugs from a well-mixed pool is viewed theoretically, and is extended to predict the time course not only of the parent drug, but also of the metabolite. The adequacy of the model is tested using phenacetin and its O-deethylated metabolite, acetaminophen.

Methods Employed: The rat liver perfused in situ preparation was employed to study the kinetics of phenacetin and acetaminophen metabolism using tracer dose quantities of these compounds. Single pass and recirculating designs were used. Separation of acetaminophen, phenacetin, and their metabolites was accomplished by an extraction procedure followed by thin layer chromatography.

Major Findings: Both acetaminophen and phenacetin were highly cleared by the rat liver preparation. However, the calculated extraction ratio of acetaminophen formed from phenacetin was less than the extraction ratio of preformed acetaminophen. These findings are consistent with the view that the deethylation of phenacetin occurred mainly in the centrilobular region while the sulfation of acetaminophen occurred throughout the liver lobule. A mathematical model has been derived based on the findings obtained with a single pass steady-state system and applied to a recirculating perfusion system. The concentrations of phenacetin and acetaminophen predicted from the model were almost identical to the values obtained experimentally.

Significance to Biomedical Research and the Program of the Institute: The present studies demonstrated some of the difficulties which may arise in predicting relationships in the kinetics of a drug and its metabolite. The phenomenon we have discovered may be important in predicting metabolite concentrations on chronic administration of a parent drug.

Proposed Course of Project: Since tracer doses of acetaminophen are converted almost entirely to its sulfate conjugate, by an enzyme in the soluble fraction of liver, it is not clear whether the phenomenon will be restricted to drugs whose metabolites are eliminated in a different organelle than they are formed. By increasing the dose of acetaminophen, the principle pathway of elimination is shifted toward glucuronide formation, a reaction that is catalyzed by an enzyme in the endoplasmic reticulum. Thus, the studies will be continued at higher doses of acetaminophen to determine whether the extraction ratio of acetaminophen derived from phenacetin is the same as that of preformed acetaminophen when acetaminophen is eliminated.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00811-01 LCP

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
A New Approach for Analyzing the Sites of Rapid Metabolism of Drugs in vivo

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

PI:	K.S. Pang	Visiting Fellow	LCP	NHLBI
	J.R. Gillette	Chief, Lab. of Chem. Pharm.	LCP	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme-Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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)

A theoretical analysis using the area under the curve of a precursor and its metabolite under different modes of drug administration was presented in an attempt to elucidate and evaluate the different sites and pathways of drug elimination during the first pass of orally administered compounds. The compounds phenacetin and acetaminophen were studied in tracer dose quantities in the rat in vivo.

Project Description:

Objectives: The "first pass" effect of orally administered drugs can be attributable to a sequence of events by eliminating organs in series. These eliminating organs or tissues are 1) the intestinal microflora, 2) intestinal mucosa, 3) liver and 4) the lung. Hepatic elimination plays a major role in drug removal during the first passage of orally administered compounds, but some drugs are known to be removed rapidly by the gastro-intestinal tract before they are absorbed into the blood stream. The objective of this project is to derive the mathematical equations for the metabolism of drugs by various organs and to evaluate the validity of the assumptions made in these derivations.

Methods Employed: Male Sprague-Dawley rats were used to study the kinetics of both phenacetin, a precursor, and its metabolite, acetaminophen. These drugs were administered either i.v., i.p. or p.o., singly or together. Blood, bile and urine were assayed for acetaminophen, phenacetin, and their metabolites by an extraction procedure followed by thin layer chromatography.

Major Findings: Theoretical analysis - Several conclusions may be made from the mathematical equations derived for a general, linear, pharmacokinetic model in which a drug is rapidly eliminated from the body by a) lung, b) kidney, c) intestinal mucosa, d) liver, and e) intestinal microflora. 1) Passive diffusion of the drug from the intestinal wall will not affect the apparent total body clearance (or the mean steady-state concentration of the drug in systemic blood) as long as the drug is not metabolized by gut microflora or eliminated unchanged with the feces. 2) Biliary secretion of the unchanged drug will not affect the mean steady-state concentration of the drug in systemic blood as long as the drug is not rapidly eliminated from the body by gastrointestinal mucosal enzymes, intestinal microflora or eliminated unchanged with the feces. 3) When the drug is rapidly eliminated by gastrointestinal mucosal enzymes, the ratio of the mean steady-state concentration of the drug in systemic blood after oral and intraperitoneal administration will depend on whether the drug given intraperitoneally is absorbed through the gut wall or through the mesentary. 4) When the drug is rapidly eliminated entirely by a single organ, and when the back diffusion of the drug from the intestine to the intestinal lumen and the biliary secretion of unchanged drug are negligible, the mean steady-state concentration of the drug is systemic blood will depend on the organ of elimination and the route of elimination. For example, after oral administration of the drug, the mean steady-state concentration of the drug in systemic blood is dependent on the intrinsic clearance of enzymes (Cl_{int}) in the gastrointestinal mucosa, liver or lung but on the tissue clearance (F Cl_{int}) of the drug eliminated by the kidney. By contrast, after administration of the drug intravenously or intramuscularly, the mean steady-state concentration of the drug in systemic blood is dependent on the intrinsic clearance (Cl_{int}) of the drug by lung but on the tissue clearance (F Cl_{int}) of the drug by gastrointestinal mucosa, liver or kidney. Because the apparent total body clearance by the lung after any of the usual routes of administration is dependent solely on

the intrinsic clearance, it is theoretically possible for the apparent total body clearance to exceed the cardiac output. 5) When the drug is eliminated solely by the liver, kidney and lung it is possible to estimate the extraction ratio of the drug as it passes through the liver from the apparent total body clearances (or areas under the systemic blood concentration curves) after intraperitoneal and intravenous administration of the drug. 6) When the drug is eliminated solely by the liver, it is possible to estimate the blood flow through the liver by measuring the areas under the systemic blood concentration curves after intraperitoneal and intravenous administration of rapidly cleared drugs. 7) When a drug is rapidly cleared by enzymes in both intestinal mucosa and liver, the apparent total body clearance after oral administration of the drug will be higher than the sum of the intrinsic clearances of the enzymes in the two tissues and after intravenous administration will be lower than the sum of the tissue clearances of the drug.

Theoretical equations were also derived for the mean steady-state concentration of drug metabolites in systemic blood. These equations revealed several other relationships. 1) When a drug is eliminated from the body by a single organ such as the liver, the areas under the plasma concentration curves of the metabolite will be identical regardless of the route of administration of the drug. 2) The area under the curve of the concentraion of the metabolite will depend on the fraction of the dose of the drug that is converted to the metabolite and on the intrinsic clearance of the metabolite by the tissue. The validity of these conclusions, however, is based on the assumption that the concentrations of unbound forms of the drug and the metabolite are the same throughout the liver and are the same as their concentrations in blood leaving the liver.

Major Experimental Findings: 1)From the area under the curve of the systemic blood concentrations of ^{3}H-acetaminophen after i.p. administration of tracer doses of ^{3}H-acetaminophen to rats, the extraction ratio, the hepatic blood flow and the intrinsic clearance of the sulfotransferase in liver were calculated. The values of the intrinsic clearance obtained from these in vivo experiments were nearly identical to those calculated from the extraction ratio and the blood flow rates of in vitro perfusion with rat liver systems. Thus the data obtained with the rat liver perfusion system appears to be valid for the metabolism of acetaminophen in rats in vivo.

Significance to Biomedical Research and Program of the Institute: The present studies would elucidate some of the complex processes which may occur on oral drug administration. It is the first attempt so far, in a comprehensive fashion, to show the different processes which may constitute the "first pass" affect. Furthermore, the studies will evaluate the validity of the assumptions made in extrapolating data from in vitro data to living animals.

Proposed Course of Project: 1) There will be other experimental designs involving administering both the precursor and its metabolite. 2) The animals will be pretreated with stimulator and inhibitors and their effects on elimination of phenacetin and acetaminophen will be quantitated. 3) Dose-dependency on phenacetin and acetaminophen will be studied. 4) The effects of a vehicle of orally administered dosage forms on the availability of phenacetin will also be investigated.

Publication:

Gillette, J.R. and Pang, K.S.: Theoretical aspects of pharmacokinetic drug interactions. Clinical Pharm. and Therapeutics, in press.

Pang, K.S. and Gillette, J.R.: Theoretical relationships between area under the curve and route of administration of drugs and their precursors for evaluating sites and pathways of metabolism. Journal of Pharmaceutical Sciences, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00812-01 LCP

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Increased Tissue Levels of Reduced Glutathione Produced by Cobaltous Chloride

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

PI:	H.A. Sasame	Chemist	LCP	NHLBI
OTHERS:	J.R. Gillette	Chief, Lab. of Chem. Pharm.	LCP	NHLBI
	M.R. Boyd	Research Associate	OD	NHLBI

COOPERATING UNITS (if any)

Dr. M. Boyd is a Research Associate in the Pharmacology-Toxicology Program, NIGMS.

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Enzyme-Drug Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

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 or chronic treatment of rats, mice and hamsters with cobaltous chloride produced up to a 200% increase in hepatic glutathione (GSH). GSH levels in lung, kidney and ileum are also markedly elevated. GSH levels are significantly elevated within 4-8 hrs after a single subcutaneous doses of 45 mg/kg of cobaltous chloride and reached maximal levels after 8 hrs. Thus, studies using cobaltous chloride as a tool to implicate cytochrome P-450 in chemical toxicities in vivo must be interpreted with caution, since GSH is known to participate in the detoxication of certain toxic, electrophilic metabolites.

Project Description:

Objectives: Administration of cobaltous chloride to animals has been known to cause a decrease in the level of hepatic cytochrome P-450, which serves as a terminal oxidase in hydroxylation of various foreign compounds. Consequently, the cobaltous chloride treatment of animals has received wide usage as a tool to implicate the in vivo participation of reactive metabolites in certain chemically induced toxicities. For example, cobaltous chloride pretreatment of rats reduced both the covalent binding and the hepatic necrosis produced by acetaminophen and bromobenzene. For example, cobaltous chloride pretreatment of rats reduced both the covalent binding and the hepatic necrosis produced by acetaminophen and bromobenzene. The positive correlation between the level of covalent binding and the degree of necrosis implicated highly reactive metabolites as the mediators of these toxicities. The known effect of cobaltous chloride on cytochrome P-450 mediated metabolism suggested that those toxic metabolites were formed by a cytochrome P-450-dependent oxidation of the parent compound. However, it is well known that the covalent binding of a reactive metabolite in vivo depends not only upon the rate of its formation but also upon the availability and/or activities of detoxication pathway for the reactive metabolite. Therefore, we have undertaken studies to examine the effect of cobaltous chloride on such potential "detoxication pathways", in order to clarify the interpretation of results employing cobaltous chloride. An additional goal of these investigations was to determine the in vivo effects of cobaltous chloride on selected toxication and detoxication pathways in extrahepatic tissues, as well as in liver.

Major Findings: 1) Pretreatment of rats with cobaltous chloride decreased the level of cytochrome P-450 not only in liver but lung and kidney, whereas much less decrease of cytochrome c reductase activities in those tissue were observed with an exception of ileum where the activities of reductase was lowered to roughly half of control values.

2) Pretreatment of rats with $CoCl_2$ raised markedly the level of reduced glutathione (GSH) in liver, lung, kidney and ileum (nearly 200%). This effect of $CoCl_2$ on GSH level was also observed with not only Sprague-Dawley rats but Fisher rats, NIH-general purpose mice and Golden Syrian hamsters.

3) Studies of dose dependency and the time course of stimulation of hepatic GSH level revealed the following: a) at the single dose of 45 mg/kg the onset time was in a range of 2-4 hrs and reached maximal after 8 hrs, b) there was a proportional increase in GSH starting at the dose level of 10 mg/kg and saturating at 45 mg/kg, c) after 64 hrs of single dose administration the elevated GSH levels at all doses of $CoCl_2$ returned to the corresponding control level.

Significance to Biomedical Research and the Program of the Institute: In view of the current concensus that chemically induced toxicities as well as cancer are closely interrelated to both the formation of P-450-mediated active metabolite(s) and detoxication of the active metabolite(s) by nucleophilic agents such as GSH, it is vitaly important to elucidate precisely the mechanism by which $CoCl_2$ or any other metabls causes the elevation of GSH *in vivo*.

Proposed Course of Project: In our continuing studies we are evaluating the effect of $CoCl_2$ on the biosynthetic pathways for GSH both *in vitro* and *in vivo*.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00813-01 LCP

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Studies on the Mechanism of Spironolactone-induced Decrease in Cytochrome P-450

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

PI:	R.H. Menard	Expert	LCP	NHLBI
OTHERS:	T. Guenther	Guest Worker	DPB	NICHHD
	H. Kon	Senior Investigator	LCP	NIAMDD
	J.R. Gillette	Chief, Lab. of Chem. Pharm.	LCP	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Enzyme-Drug Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.40	0.40	

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 spironolactone (100 mg/kg, i.v.) to dogs causes a decrease of 40-50% within 4 to 5 hr in the adrenal or testicular content of the activities of steroid hydroxylases, thereby resulting in a 40-50% lowering of the plasma concentration of testosterone, estradiol and cortisol. The loss of the steroid hydroxylase activities by spironolactone stems from a specific destruction of the heme of cytochrome P-450 resulting in the rapid degradation of its apoproteins. Studies with several analogues of spironolactone have shown that the -SH group at the carbon-7 position of the steroid moiety is required for the loss of adrenal or testicular steroid hydroxylases. In vitro experiments with micorsomal suspensions have indicated: 1) that spironolactone can be activated to cause a destruction of P-450 and its heme without a loss of the apoprotein, 2) that the activation requires NADPH, O_2 and an active P-450 and 3) that the loss of P-450 can be prevented by addition of progesterone or by the conversion of P-450 to its inactive form P-420.

Project Description:

Objectives: To determine the mechanism by which spironolactone causes an impairment in steroid biosynthesis in the adrenal and testis.

Methods Employed: Enzymatic activity of various steroidogenic enzymes was determined in the microsomal fraction of cells isolated from the adrenals or testes of various species following spironolactone administration, or in adrenal or testicular microsomal suspensions in which spironolactone and/or spironolactone analogues were added. Spectra of the cytochrome P-450 was determined either optically or by electron spin resonance. Apoprotein bands of cytochrome P-450 were separated by SDS gel-electrophoresis and the concentrations determined optically.

Major Findings: The intravenous administration of spironolactone (100 mg/kg) to dogs causes a decrease of 40 to 50% within 4 to 5 hr of the adrenal or testicular content of steroidogenic enzymes involved in the hydroxylation of physiological steroids which, in turn, results concomitantly in a 40 to 50% lowering of the plasma concentration of testosterone and estradiol in the spermatic vein, and of cortisol in the adrenal vein. Previous findings have indicated that spironolactone treatment causes a specific destruction of the heme of cytochrome P-450, the active site of steroid hydroxylases, as first measured by the pyridine haemochromogen method and recently confirmed by electron spin resonance measurements. Moreover, the in vivo loss of the P-450-heme is concurrent with a similar loss in the concentration of the apoproteins of cytochrome P-450 associated with steroid hydroxylases. The latter finding suggests that a loss of the prosthetic group of P-450 following spironolactone treatment results in the rapid degradation of its apoprotein.

Deacetylation or hydrolysis of the thioacetate group of spironolactone produces a thio-steroid analogue which when administered in vivo (100 mg/kg, i.v.) produces effects identical to those of spironolactone. It causes a loss of adrenal or testicular content of steroid hydroxylases and a decrease in the plasma level of steroids. Moreover, like spironolactone, the thio-analogue causes no adrenal or testicular histological changes and the loss of enzymatic activity is reversible within 3 to 4 days. The administration of aldadiene (100 mg/kg, i.v.) a metabolite of spironolactone that does not contain the thioacetate group, causes no loss in t e content of adrenal or testicular steroid hydroxylases and no decrease in the plasma concentration of estradiol or testosterone. Thus, the above results indicate that the -SH group of the carbon-7 position of the steroid moiety is required for the in vivo loss of adrenal or testicular steroid hydroxylases.

In the testis, the loss of steroid hydroxylases occurs in the cells of Leydig which contain as much as 90% of the testicular cytochrome P-450. However, in the adrenal, the spironolactone-induced loss of hydroxylase activity and/or cytochrome P-450 is first detected in the zona fasciculata

and then in the zona glomerulosa. The latter observation supports our hypothesis that spironolactone is activated to a metabolite by cells that have a high activity of steroid 17α-hydroxylation since no loss of steroid hydroxylase activity occurs in steroid-producing cells that have a low steroid 17α-hydroxylation activity such as cells from the zona glomerulosa when separated from zona fasciculata of cortisol-secreting animals, or adrenals from corticosterone producing animals.

In vitro experiments with microsomal suspensions prepared from adrenals or testes have indicated the following: (1) that spironolactone or its thio-analogue but not aldadiene can be activated to cause a destruction of cytochrome P-450 and its heme and cannot be prevented by the addition of glutathione or DTT, (2) that both spironolactone and its thioanalogue cause type I spectral changes and activation requires NADPH, oxygen and an active cytochrome P-450, (3) that the breakdown of P-450 by the thioanalogue can be prevented by the addition of progesterone or by the conversion of P-450 to its inactive form, cytochrome P-420, without a loss of substrate binding, (4) that an in vitro loss of the heme of P-450 does not result in an in vitro loss of its apoprotein, (5) that a destruction of P-450 by spironolactone also occurs in microsomal suspensions prepared from human adult or fetal adrenals and only in the adrenal steroid-producing cells of the zona fasciculata and (6) that ESR measurements have indicated a heme loss of P-450 without the appearance of new ESR signal for protoporphyrin heme suggesting that the sulfur atom from the steroid moiety is not incorporated into the pyrrole ring of the porphyrin.

Significance to Biomedical Research and to the Program of the Institute: Spironolactone is the first example of a steroidal drug which is able to cause a loss of steroid hydroxylation, and is also the first drug shown to cause such a decrease in endocrine tissues. Some of the adverse effects of spironolactone treatment in man are gynecomastia, impotence, loss of libido and amenorrhea. Such effects of spironolactone administration may be related to its inhibitory effect on androgen and estrogen biosynthesis. Thus a study of the mechanism by which spironolactone causes a loss of cytochrome P-450 in adrenal or testicular tissues represents an initial step toward understanding how the action of drugs, such as spironolactone, may alter the biosynthesis of physiologically active steroids in endocrine tissues.

Proposed Course of Project: To expand the above studies in order to elucidate the mechanism of the specificity of spironolactone for the apoprotein of adrenal or testicular 17α-hydroxylase.

Publications: None

PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

Z01 HL 00814-01 LCP

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Spironolactone Inhibition of Mitotane-induced Adrenal Atrophy and Necrosis

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

PI:	R.H. Menard	Expert	LCP	NHLBI
OTHERS:	G.B. Cutler	Clinical Associate	RRB	NICHHD
	S. Rifka	Clinical Associate	RRB	NICHHD
	J.R. Gillette	Chief, Lab. of Chemical Pharm.	LCP	NHLBI
	F.C. Bartter	Chief, Hypertension-Endocrine Branch	HE	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme-Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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)

Daily administration of o,p'-DDD (50 mg/kg,o.p.) to dogs for two weeks resulted in adrenal atrophy, necrosis, and subsequent loss in the content of adrenal cytochrome P-450. When a single dose of o,p'-DDD (50 mg/kg) was administered intravenously, moderate necrosis of the cells in the zona fasciculata occurred within 4 to 5 hr and was characterized enzymatically by a 30-50% loss in the activity of mitochondrial and microsomal cytochrome P-450 enzymes. The latter effects of o,p'-DDD treatment were prevented when the dogs were pretreated with an intravenous dose of spironolactone (100 mg/kg). The studies with spironolactone, which depletes the adrenal content of cytochrome P-450 dependent enzymes, suggest that oxidation or hydroxylation of o,p'-DDD by a cytochrome P-450 dependent enzyme may be prerequisite for adrenal necrosis to occur.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To study the mechanism by which o,p'-DDD (Mitotane) causes adrenal atrophy and necrosis in dog and man.

Methods Employed: Following treatment of dogs with either o,p'-DDD and/or spironolactone, adrenals were removed and various activities of mitochrondrial or microsomal enzymes were assayed and correlated with histological changes. In vitro binding of o,p'-DDD to adrenal estrogen and androgen steroid receptors were determined by competitive studies.

Major Findings: Daily administration of o,p'-DDD (50 mg/kg, o.p.) to dogs for two weeks resulted in adrenal atrophy, necrosis, and subsequent loss in the content of microsomal and mitochondrial cytochhrome P-450. When a single dose of o,p'-DDD (50 mg/kg) was administered intravenously, moderate necrosis of the cells in the zona fasciculata occurred within 4 to 5 hr and was characterized histologically by a marked loss of the cord-like arrangement of the cells and of the pycnosis of nuclei, and was characterized enzymatically by a 30 to 50% loss in the activity of mitochondrial and microsomal cytochrome P-450 enzymes. The latter effects of o,p'-DDD treatment were prevented when the dogs were pretreated with an intravenous dose of spironolactone (100 mg/kg). In addition, the necrosis occurred by o,p'-DDD treatment did not result from a possible estrogenic or antiadrogenic effect since o,p'-DDD did not interfere with the in vitro binding of tritiated estradiol to the adrenal estrogen receptor or of tritiated dihydrotestosterone to the adrenal androgen receptor.

Significance to Biomedical Research and to the Program of the Institute: Because o,p'-DDD is selectively toxic only to the adrenal cortex of dogs and human, the drug is of value for treatment of inoperative adrenocortical carcinoma or of Cushing's Syndrome. Knowledge on the mode of action or biotransformation of o,p'-DDD is limited. The above studies with spironolactone, which depletes the adrenal content of cytochrome P-450-dependent enzymes, suggest that oxidation or hydroxylation of the drug by a cytochrome P-450-dependent enzyme may be a prerequisite for adrenal necrosis to occur.

Proposed Course of Project: To determine the mechanism by which o,p'-DDD is activated to a toxic metabolite in the adrenal gland of dogs and humans.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00815-01 LCP

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Assay of Cortisol in Human Plasma by High Pressure Liquid Chromatography (HPLC)

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

PI:	R.H. Menard	Expert	LCP	NHLBI
OTHERS:	S. Nelson	Staff Fellow	OD	NHLBI
	G. Dallas	Lab. Director		

COOPERATING UNITS (if any)

Dr. S. Nelson is a Staff Fellow in the Pharmacology-Toxicology Program, NIGMS and Dr. George Dallas is a Laboratory Director at Dupont Instrument, Wellington, DElaware.

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Enzyme-Drug Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

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)

A method for the assay of cortisol in human plasma has been developed by HPLC that incorporates the separation of interfering compounds, and that is rapid (30 to 35 min), practical, and requires only 500 µl of plasma.

Project Description:

Objectives: To develop a simplified technique for the detection and quantitation of cortisol in human plasma that incorporates the separation of interfering compounds and the measurement of cortisol.

Method Employed: Dichloromethane was used to extract cortisol from an aliquot of human plasma. The dichloromethane extract of plasma was chromatographed by high pressure liquid chromatography on a Zorbax SIL column with a solvent system of dichloromethane and methanol. Cortisol was monitored by absorbance at a wave length of 254 mμ. Tritiated-cortisol was added to plasma samples to correct for procedural loss after HPLC separation. The identity of cortisol was confirmed by radioimmunoassay and by mass spectrometry.

Major Findings: Retention time of cortisol on a SIL column was 9.4 min. Different retention times were noted for 17 various steroids and/or drugs structurally related to cortisol. The area of the peak detected by absorbance at 254 mμ corresponded to cortisol concentrations varying between 25 to 80 nanograms; the sensitivity of detection was 2 to 5 ng of cortisol.

Quantitation of cortisol by HPLC was similar to values obtained when the cortisol fraction after HPLC was collected and determined by radioimmunoassay. Cortisol in the HPLC fraction was identified by mass spectrometry.

Significance to Biomedical Research and to the Program of the Institute: A number of methods have been described for the determination of plasma or urine cortisol. The methods in current use are the fluorimetric, the radioligand assay using plasma rich in cortisol-binding-globulin, and the radioimmunoassay. In many instances, the latter methods must employ a chromatographic step for an accurate measurement of plasma or urine cortisol due to interfering drugs or steroids. For example, the presence of spironolactone in plasma causes an increase in the fluorescent measurement of cortisol, or causes a displacement of cortisol from its antibody in the radioimmunoassay. Thus, treatment of man with spironolactone appears to increase the concentration of plasma cortisol when measured by the usual methods. However, the present HPLC method which incorporates the separation of spironolactone and/or its metabolites from cortisol indicates that spironolactone treatment may actually decrease the concentration of blood cortisol. Moreover, with HPLC a patient's plasma cortisol can be measured and determined within 30-35 min, whereas other methods require at least 1 to 2 hr. Thus, HPLC provides a technique for the measurement of plasma cortisol that is rapid, practical and requires only 500 μl of plasma.

Proposed Course of Project: Terminated.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00816-01 LCP

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Loss of C_{17}-C_{20} Lyase and Ovarian 17α-Hydroxylase Activities by Spironolactone

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

PI:	R.H. Menard	Expert	LCP	NHLBI
OTHERS:	H. Borner	Visiting Fellow	LCP	NHLBI
	D. Madison	Staff Fellow	LCP	NCI
	J.R. Gillette	Chief, Lab. of Chemical Pharm.	LCP	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Enzyme Drug Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland

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)

The adimistration of spironolactone (100 mg/kg) caused a 40 to 60% loss in the activity of the C_{17}-C_{20} lyase in adrenals and testes of guinea pigs and dogs, and caused a 30 to 40% decrease in the ovarian activity of the 17α-hydroxylase in rats.

Project Description:

Objectives: To determine whether the administration of spironolactone may alter steroid 17α-hydroxylase activity in the ovarian gland, and also steroid C_{17}-C_{20} lyase activity in the adrenal and testis.

Methods Employed: Rats and guinea pigs were treated intraperitoneally with spironolactone at a dosage of 100 mg/kg for 3 days, whereas dogs were given an intravenous dose of spironolactone (100 mg/kg) for 4 to 5 hr. Enzymatic assays were determined by measuring the microsomal rate of conversion of labeled progesterone to labeled 17α-hydroxyprogesterone or of labeled 17α-hydroxyprogesterone to labeled androstenedione.

Major Findings: The administration of spironolactone caused a 40 to 60% loss in the activity of the C_{17}-C_{20} lyase in adrenals and testes of guinea pigs and dogs, and caused a 30 to 40% decrease in the ovarian activity of the 17α-hydroxylase in the rat.

Significance to Biomedical Research and to the Program of the Institute: Spironolactone is an aldosterone antagonist often used in the treatment of primary aldosteronism, low renin essential hypertension, and disorders of spontaneous or iatrogenic hypokalemia. The clinical usefulness of spironolactone, however, is limited by its adverse side effects which include decreased libido and impotence in men, and menstrual irregularity in women. Previous studies have shown that the administration of spironolactone causes a chronic decrease in steroid 17α- and 21-hydroxylation in the adrenal and testis. In the present study, evidence is presented indicating that spironolactone treatment may also cause a decrease in the activity of adrenal or testicular steroid, C_{17}-C_{20} lyase, a major enzyme in the steroidogenic pathway of the endoplasmic reticulum. A decrease in the latter pathway of steroid interconversion in the cell correlates with a decrease in the concentration of plasma testosterone, estradiol, and cortisol.

Menstrual irregularity induced by spironolactone treatment may be explained by a decrease in ovarian 17α-hydroxylase activity. For example, in congenital 17α-hydroxylase deficiency the ovary contains only primordial follicles, suggesting that a deficiency of this enzyme leads to chronic anovulation.

Proposed Course of Project: Terminated

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00817-01 LCP

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Studies on Aryl Hydrocarbon (Beno(a) Pyrene) Hydroxylase (AHH) in the Adrenal

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

PI:	R.H. Menard	Expert	LCP	NHLBI
OTHERS:	T. Guenther	Guest Worker	DBP	NICHHD
	J.R. Gillette	Chief, Lab. of Chem. Pharm.	LCP	NHLBI
	D. Nebert	Chief, Developmental Pharm. Branch	DPB	NICHHD

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Enzyme-Drug Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

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)

Of the species examined, the rat, a corticosterone-producing animal, was found to have the highest specific activity of AHH in the adrenal gland, whereas a low or no activity was found in three adult cortisol-producing animals, the guinea pig, dog, and man. In the rat adrenal, AHH activity was localized predominantly in the zona fasciculata, and was not inducible by 3-methyl cholanthrene or TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) treatment, but only by ACTH administration. Following ACTH treatment of rats, the increase in AHH activity was concurrent with a similar increase in the concentration of an apoprotein of cytochrome P-450 in the molecular weight region of 54,000. The latter apoprotein of P-450 was absent in the adult but not the fetal human adrenal and its absence correlated with the lack of AHH activity, but not steroid hydroxylase activity. The latter finding suggests that the P-450 apoprotein of 54,000 molecular weight may be specific for AHH activity.

Project Description:

Objectives: To study the mechanism by which AHH activity is regulated in the adrenal gland.

Methods Employed: Benzo(a) pyrene hydroxylase activity was assayed in various subfractions of cortisol- or corticosterone-producing adrenals of either nontreated animals or animals treated with various inducers of steroid or drug metabolism. Spectrophotometric methods were used to determine the concentrations of P-450 and apoproteins; the latter after separation by SDS gel electrophoresis.

Major Findings: In the adrenal gland, AHH activity appears to be localized only to the microsomal fraction of the cells; little or no activity was detected in the mitochondrial fraction even though the level of cytochrome P-450 is twice that of the microsomes. Of the species examined, the rat, a corticosterone-producing animal, was found to have the highest specific activity of AHH, whereas a low or no activity was found in three adult cortisol-producing animals -- the guinea pig, dog, and man. In man, the fetal adrenal contains both AHH and steroid hydroxylase activities, whereas only steroid hydroxylase activity was detected in the adult adrenal. The absence of AHH activity in the adult adrenal could be correlated with the absence of a specific apoprotein band of P-450 in the molecular weight region of 54,000; the latter band was present in the fetal adrenal.

In the rat adrenal, 8 to 10% of the total activity of AHH was localized in the zona glomerulosa, whereas 70 to 80% was found in the zona fasciculata.

In hypophysectomized rats, adrenal AHH apoprotein and/or activity decreased 2 to 20% of control animals. Intraperitoneally treatment of hypophysectomized or normal rats either with a single dose of 3-methylcholanthrene (80 mg/kg) for 48 hr or with TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) at a dosage of 40 µg/kg for 10,4, or 2 days caused no increase in benzo(a) pyrene or steroid hydroxylase activity or in the concentrations of the apoproteins of P-450. Treatment of hypophysectomized rats with ACTH (8 units, 2x daily, s.c.) caused a 10 to 13-fold rise in the AHH activity which correlated with a similar rise in the concentration of an apoprotein of P-450 in the molecular weight region of 54,000.

Significance to Biomedical Research and to the Program of the Institute: Because of their potential as carcinogens, polycyclic hydrocarbons represent a group of substrates for membrane-bound mono-oxygenases. The activity of one of these enzymes, aryl hydrocarbon (benzo(a) pyrene) hydroxylase (AHH) is increased in hepatic tissue by the in vivo administration of polycyclic hydrocarbons, thereby increasing the rate of metabolism of chemical carcinogens. In the rat adrenal, the activity of AHH is not substrate inducible by polycyclic hydrocarbons, but can be altered by the pituitary hormone, ACTH. In man, the absence of AHH activity in the adult but not the fetal adrenal can be correlated with the loss of a specific apoprotein of cytochrome P-450.

The latter findings suggest that the apoprotein for AHH activity in the human adrenal may be associated specifically with the metabolism of polycyclic hydrocarbons such as benzo(a) pyrene, and not with the metabolism of steroids such as progesterone.

Proposed Course of Project: To study the biochemical properties of AHH activity in the adrenal gland of the human fetus and adult.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00826-01 LCP

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Mechanism of the In Vivo Irreversible Binding of Chloramphenicol

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

PI:	L.R. Pohl	Senior Staff Fellow	LCP	NHLBI
	G. Krishna	Chief, Section on Drug-Tissue Interaction	LCP	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Drug-Tissue Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.6	

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)

The mechanism of the in vivo irreversible binding to tissue protein of [^{14}C] chloramphenicol was investigated by determining the chemical nature of its bound metabolites. By employing several techniques such as Sephadex and tlc chromatography, chemical and enzyme hydrolysis, and reverse isotope dilution analysis, it was found that the majority of the [^{14}C] bound material consisted of approximately 50% glycine and 50% serine. Nearly an identical result was obtained when the plasma protein from rats administered [^{14}C] dichloroacetate acid was investigated. These findings establish that CAP and dichloroacetic acid are metabolized by a similar route to yield two carbon units. These fragments are converted into glycine and serine and subsequently incorporated into protein. This route of metabolism may represent a minor but a unique detoxifying process since it appears to involve the hydrolytic cleavage of the chlorines from CAP. These chlorines have previously been shown to be structural requirements for the in vitro activation of CAP into a reactive acylating agent.

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Project Description:

Objectives: Previous studies from this laboratory have indicated that CAP is activated in vitro and in vivo to reactive intermediates that bind covalently to various macromolecules. The in vitro activation of CAP by rat microsomes appears to involve an oxidative dechlorination process to yield an oxamyl chloride reactive intermediate which reacts covalently with microsomal protein. A different process appears to predominate in vivo since the bound metabolites contain radiolabel that was derived primarily from the dichloroacetyl portion of CAP. The objective of the present investigation has been to determine the mechanism of this in vivo biotransformation pathway. It is hoped that this information will lead to a more complete understanding of the metabolism of CAP and potentially how this compound produces bone marrow depression.

Methods Employed: Either [^{14}C] CAP or [^{14}C] dichloroacetic acid (100 μmole/kg) was administered intraperitoneally to Sprague-Dawley male rats (200 g), which were pretreated for 3 days with phenobarbital (80 mg/kg,i.p.). After 24 hr, the plasma from the animals was collected and subjected to the following analysis: 1) Sephadex G-200 column chromatography, 2) enzymatic hydrolysis with Pronase followed by Sephadex G-10 column chromatography, 3) acid hydrolysis followed by extraction analysis, 4) tlc of acid hydrolyzates, and 5) reverse isotope dilution of acid hydrolyzates with glycine and serine.

Major Findings: The [^{14}C] bound metabolites of CAP and dichloroacetic acid behaved nearly identical during each analytical procedure. 1) Sephadex G-200 Chromatography. Each fraction of plasma protein eluting from the column contained radiolabel. 2) Pronase Hydrolysis and Sephadex G-10 Chromatography. Three radioactive fractions eluted from the columns after Pronase hydrolysis. 3) Acid Hydrolysis and Extraction Analysis. The hydrolyzates were found to be zwitterions and therefore appeared to be amino acids. 4) Tlc Analysis of Acid hydrolyzates. Two radioactive fractions were detected on the tlc plates. One fraction had the same R_f as glycine, whereas the other had the same R_f as serine. 5) Reverse Isotope Dilution with Glycine and Serine. This analysis revealed that glycine and serine were virtually the only radiolabeled products in the acid hydrolyzates. They were present in approximately equal amounts.

The results of the present investigation indicate that the majority of the in vivo binding to tissue protein of CAP and dichloroacetic acid is not due to bioactivation and covalent binding of a reactive metabolite. Instead, the binding appears to be due to the conversion of a common metabolite of these two compounds into glycine and serine. These amino acids are then incorporated into tissue protein.

This metabolic pathway differs significantly from the process that occurs in vitro with liver microsomes. Under these conditions, CAP is activated into an oxamyl chloride metabolite by an oxidative dechlorination mechanism.

This reactive intermediate reacts with microsomal protein to yield a covalently bound product. In vivo, other processes appear to compete with this bioactivation mechanism. The conversion of CAP and dichloroacetic acid into glycine and serine must involve a nonoxidative pathway of dechlorination. A likely mechanism would involve the hydrolysis of the dichloromethyl carbon into an aldehyde. After hydrolysis of the amide bond of CAP, the resulting product would be glyoxylic acid; the same product formed from the hydrolytic dechlorination of dichloroacetic acid. Glyoxylic acid is known to be transaminated into glycine. And glycine is known to be converted into serine.

Significance to Biomedical Research and Program of the Institute: The hydrolytic dechlorination mechanism may potentially represent a detoxifying process. If CAP were dechlorinated by this pathway, it no longer can be bioactivated by the oxidative dechlorination mechanism into a reactive and potentially toxic metabolite. In the overall process of detoxification of CAP it may only represent a minor pathway (less than 10%) but it may have greater significance in the metabolism of thiamphenicol.

Proposed Course of Project: The study of the mechanism of the in vivo binding of CAP in the rat is now completed. We plan to investigate the nature of the hydrolytic dechlorination process. We feel that this metabolic pathway may be a detoxifying mechanism not only for CAP, thiamphenicol, but also for other halogenated hydrocarbons such as halothane.

Publications:

Pohl, L.R., Nelson, S.D. and Krishna, G.: Investigation of the mechanism of the metabolic activation of chloramphenicol by rat liver microsomes: Identification of a new metabolite. Biochem. Pharmac., in press.

Gillette, J.R., and Pohl, L.R.: A prospective on covalent binding and toxicity. J. Toxicol. Environ. Health 2: 849-871, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00830-03 LCP

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

The Role of Calcium and Cyclic GMP in the Secretion of Amylase from Pancreas

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

PI:	C.L. Kapoor	Visiting Fellow	LCP	NHLBI
	G. Krishna	Chief, Section on Drug-Tissue Interaction	LCP	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Pharmacology

SI
Drug-Tissue Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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)

Earlier we have reported that cGMP is involved as a mediator in the stimulus-secretion coupling in pancreas and flux of calcium is intimately linked with this process. In this study we have demonstrated that calcium can activate guanylate cyclase in a cell-free system. It has been found by immunocyto-chemical technique that cGMP is localized in the lumen, apical plasma lemma membrane and zymogen granules and possibly secreted by a process of exocytosis. Localization of cAMP in cytoplasm of acinar cell did not change significantly. From these studies it appears that calcium may be the mediator for the increase in cGMP and that cGMP along with Ca^{++} may participate in the process of exo-cytosis.

Project Description:

Objectives: One of the initial events in the pancreatic acinar cells after interaction with various secretagogues is a marked depolarization of cell membrane. Previously we reported that the secretagogues increase the concentration of cGMP rather than that of cAMP and that they increased the efflux of calcium. Since cytoplasmic calcium is presumably increased markedly during this process, we tested its effect on the activation of guanylate cyclase, the enzyme involved for the synthesis of cGMP. Moreover, by fluorescent immunocytochemical techniques the sequence of events was determined during the accumulation and secretion of cGMP and amylase caused by various secretagogues.

Methods Employed: Hartley guinea pigs (Male NIH strain) weighing 250-300 g were fasted over night. Pancreatic lobules were prepared according to the procedure of Scheele and Palade (J. Biol. Chem. 250:2660, 1975). The lobules were incubated at 37°C for 30 min in Krebs Ringer bicarbonate buffer pH 7.4, containing 1 mg/ml glucose. The medium was decanted and the lobules were washed and resuspended in the same medium. About 4 to 8 lobules weighing 20-40 mg were incubated with carbamylcholine, caerulein and other agents in 2 ml of Krebs Ringer bicarbonate buffer (pH 7.4) saturated with 95% O_2 - 5% CO_2 in Dubnoff metabolic shaker at 37°C for various times. At the end of the incubation period, the medium was removed and frozen and lobules remaining in the flask were rapidly frozen in liquid nitrogen or dry ice. A portion of the frozen medium was heat denatured and used for cyclic nucleotide determination. The cyclic nucleotides present in the lobules were extracted by homogenization of tissue in 5% TCA. The extract was centrifuged. TCA was removed by ether extraction and ether was removed by incubating samples at 50°C. The cAMP and cGMP were assayed by the radioimmunoassay method of Frandsen and Krishna (Life Science 18:529, 1976). Amylase released into the medium was assayed according to the method of Rinder Knecht et al.,Experientia 23:805 (1967), amylase azure being used as substrate.

In order to determine the cytochemical localization of cyclic nucleotides, pieces of pancreas were incubated in Krebs Ringer buffer pH 7.4 for various periods of time as described above. At the end of the incubation period, pieces of pancreas were removed from the medium and frozen immediately on a cardboard pad by immersion in 2 methyl butane (Eastman Kodak). They were then cooled in liquid nitrogen and sections of 4 to 6 μ thick were cut in cryostat microtome (Slee International). These sections were transferred to cover slip and dried. An antiserum to cGMP and cAMP were raised in rabbits as described by Steiner et al. (J. Biol. Chem. 247:1106, 1972) and were diluted to 1:8 and 1:10, respectively with phosphate buffered saline (PBS). The fluorescent immunocytochemical localization of cyclic nucleotides were carried out under a Zeiss dark field fluorescence microscope through a fluorescence isothiocynate filter for a fixed time as described by Kapoor and Krishna (Science 196:1003-1005,1977).

The soluble and total particulate fraction of guinea pig pancreas were prepared by homogenization of tissue in (10 volumes of 100 mM Tris HCl buffer

(pH 7.4) at 4°C in a glass homogenizer with a Teflon pestle. The homogenate was centrifuged at 109,000 x g for 1 hr. Particulate fractions were resuspended in the original volume of Tris HCl buffer. Guanylate cyclase was assayed as described previously according to the procedure of Krishna and Krishnan (J. Cyclic Nucleotide Res. 1, 293-302, 1975).

Major Findings: 1) Carbamylcholine (30μM) and caerulein (3nM) increased the concentration of cGMP in guinea pig pancreatic lobules about 8 to 10-fold over the basal within 30 sec with concomitant increase in rate of amylase secretion. The concentration of cGMP declined to a value about 16% of the peak level in 10 min and rose again to a constant value of 60% of peak level. Cellular cGMP decreased because the nucleotide was secreted into the medium. Cellular cAMP, however, did not change, nor was this nucleotide secreted into the medium. 2) Dibutyryl cGMP caused a 2-fold increase in the rate of amylase secretion, while the calcium ionophore, A 23187 which caused a marked increase in calcium flux induced only a 2-fold increase in amylase secretions. Since various secretagogues induce both calcium efflux and cGMP accumulation. It could be inferred from these studies that both calcium and cGMP are essential for optimal amylase release from pancreas. 3) In guinea pig pancreas more than 80% of guanylate cyclase was present in the soluble fraction. The soluble enzyme had a K_m of 50 μM, when assayed at 3 mM Mn^{++}. The soluble guanylate cyclase was activated more than 400% by 5 mM of calcium when assayed at 1 mM of GTP and submaximal concentrations of Mn^{++} (0.3 - 1 mM). Moreover, low concentrations of calcium (10-100 μM) stimulated the enzyme activity more than 50% when assayed at low GTP and Mn^{++} concentrations (0.1 mM). The effect of various secretagogues on soluble and particulate guanylate cyclase was investigated. Carbamylcholine, caerulein or cholecystokinin octapeptide at concentrations which caused maximal increase in cGMP and amylase secretion did not activate pancreatic guanylate cyclase in cell-free systems. Even by increasing these concentrations 100-fold it did not produce any effect. These agents caused dose-dependent increase in calcium efflux which correlated with increases in cGMP and the amylase secretion. These results suggest that various secretagogues induced cGMP accumulation by mobilization of internal stores of calcium which activate guanylate cyclase. The mechanism by which cGMP induces amylase secretion, however, remain to be elucidated. 4) A fluorescence immunocytochemical technique showed that cGMP was distributed in the apical plasma lemma membrane and lumen of pancreas. Carbamylcholine (30 μM) increased the cGMP fluorescence in the apical plasma lemma membrane within 30 sec and in zymogen granule and plasma membrane in the apical part of acinar cells in 10 min. However, the cells in the islets of Langenhan which are involved in the endocrine secretion of glucagon and insulin did not show any significant change in cGMP immunofluorescence. Concentration of cAMP in the resting and stimulated pancreatic acinar cells were not altered and cAMP was diffusely localized along the apical portion of plasma lemma membrane and cytoplasm.

Significance to Biomedical Research and to the Program of Institute: The elucidation of the regulation of cGMP in the secretory process provides a new understanding into the initial events of the stimulus-secretion coupling of exocrine pancreas. Moreover, these studies raise the possibility that

similar mechanism(s) may be involved in other secretory processes including the secretion of surfactant from lung.

Proposed Course of Project: Studies will be carried out utilizing biochemical and immunocytochemical techniques to explore the molecular mechanism(s) by which these cyclic nucleotides are involved in other secretory processes such as histamine release from mast cells, catecholamine release from adrenal medulary cells and surfactant secretion from lung type II alveolar epithelial cells will be attempted.

Publications:

Kapoor, C.L. and Krishna, G.: Hormone-induced cyclic GMP secretion from guinea pig pancreatic lobules. Science 196:1003-1005, 1977.

and

Krishna, G., Krishnan, N., Fletcher, R.T.,/Chader, G: Effects of light on cyclic GMP metabolism in retinal photoreceptors. J. Neurochemistry 27: 717-722, 1976.

Frandsen, E.D. and Krishna, G.: A simple ultrasensitive method for the assay of cyclic AMP and cyclic GMP in tissues. Life Sciences 18: 529-542, 1976.

Christophe, J., Frandsen, E.K., Conlon, T.P., Krishna, G. and Gardner, J.D.: Action of cholecytokinin, cholinergic agents and A 23187 on accumulation of guanosine 3':5'-monophosphate in dispersed guinea pig pancreatic acinar cells. J. Biol. Chem. 261: 4640-4645, 1976.

PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00831-02 LCP

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Chloramphenicol-induced Aplastic Anemia

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

PI:	G. Krishna	Chief, Section on Drug-Tissue Interaction	LCP	NHLBI
	I. Aykac	Guest Worker	LCP	NHLBI
OTHERS:	G.B. Reddy	Vet. Medical Officer	BUM	FDA
	N.R. Hayes	Vet. Medical Officer	VRB	DRS

COOPERATING UNITS (if any)
Bureau of Vet. Medicine, Food and Drug Administration and Veterinary Resources Branch, Division of Research Resources, NIH.

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Drug-Tissue Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20014

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)

Chloramphenicol-induced aplastic anemia in calves by oral administration of the drug appears to require the presence of either the nitro group or the dichloroacetamide group. Modification of the drug molecule by replacement of either nitro group by methyl sulfonyl group (as in thiamphenicol) or the dichloroacetamide group by trifluoroacetamide group (as in fluoramphenicol) lowers the incidence but does not prevent the toxic effect. The replacement of both nitro group by methyl sulfonyl group and dichloroacetamide group by trifluoracetamide group (as in trifluorothiamphenicol) appears to eliminate the toxic effect of the drug.

Project Description:

Objectives: Last year we reported that chloramphenicol induces aplastic anemia in calves by oral administration of the drug. One of the main objectives of this study has been to show whether by chemical modification of the chloramphenicol molecule if we could modify or eliminate this potential toxicity of chloramphenicol on bone marrow.

Methods Employed: Seventeen Holstein calves weighing 90-100 kg of both sexes have been utilized in the study. The calves were fed ad libidum calf chow and had free access to water. Blood samples were collected daily for 5 days before the initiation of the experiment and total cell counts, including differentials that were established. Chloramphenicol, thiamphenicol, trifluorochloramphenicol and trifluorothiamphenicol (100 mg/kg/day) were administered for 10 days in gelatin capsules. Animals were sacrificed at the end of 6-7 weeks after the start of the experiment. During the trial, weekly blood samples were collected in tubes containing EDTA and a differential cell count was made. Before the calves were killed, bone marrow biopsies from the sternum were obtained. Bone marrow smears and bone marrow sternal biopsies were placed immediately in absolute methanol. Portions of the ribs from Costo-condral junction, sternum and femoral head were collected and fixed in buffered formalin or methanol. Sections of liver, lymph nodes, spleen, kidney, thymus and adrenals were fixed, embedded, sectioned and stained. Blood smears were fixed in methanol and stained with Geimsa, Wright, Mary Greenwald Geimsa for cellular morphology and brilliant cresol for reticulocyte vital staining. Bone smears fixed in methanol were stained with any of the above methods to determine ty Cytological Characteristics of the bone marrow cells. Bone marrow sections and smears were also stained to reveal the free iron. Nucleoproteins and their precursors in blood cells were stained by methyl green pyronin stain. In order to differentiate lymphocytes from other leucocytes, histochemical localization of peroxidase was employed. Cytoplasmic vasculization and overt oil droplets in bone marrow cells were demonstrated by staining the smears with Sudan IV and Oil Red O.

Paraffin sections of bone marrow were assayed for peroxidase, RNA, DNA and free iron. Sections of other tissues were stained with hemotoxylin and eosin.

Major Findings: Similar results were obtained with all of the analogues of chloramphenicol. There were no gross lesions, except for hyperimia of sinus membranes. However, vacuolization of the proerythroblasts were seen in bone marrow sections and in smears and there were highly localized areas of aplasia in bone marrow sections.

In the kidneys there was a dilatation and a hyperemia of vessels in the capsular region and the glomerular vascular network. Similar dilations and hyperemia of vessels were noted in liver. Hyperemia of capsule and sinus enlargement in the peripheral and travecular regions was noticed in lymph nodes.

The blood cell counts including the differential did not change during the course of the treatment with any of the drugs. However, with chloramphenicol and triamphenicol there was predominance of lymphocytes in comparison to untreated calves.

All the analogues of chloramphenicol containing either nitro group or dichloroacetamide group produced characteristics similar to aplasia in the bone marrow of calves. These include chloramphenicol, thiamphenicol and trifluorochloramphenicol. The extent of lesion was greater in chloramphenicol than its trifluoro-analogue or thiamphenicol. The analogue containing neither a nitro group nor a dichloroacetamide group (namely trifluorothiamphenicol) appeared to produce no lesions in bone marrow in two calves so far tested.

Significance in Biomedical Research and to the Program of the Institute: The finding that chloramphenicol in calves causes lesions similar to aplastic anemia has prompted the synthesis of various other analogues of chloramphenicol which may have the antibiotic activity without causing serious side effects such as aplastic anemia.

Proposed Course of Work: It is known that the incidence of aplastic anemia caused by chloramphenicol in humans is smaller when the drug is administered parenterally than when it is administered orally. With the availability of the calf model we propose to compare the incidence of toxicity caused by chloramphenicol and its analogues after parenteral and oral administration. Since it appears that both nitro group and the dichloroacetamide group in the chloramphenicol molecule are responsible for induction of aplastic anemia in calves it should be interesting to test whether nitro reduction by the bacterial flora may be responsible for the aplastic anemia.

Publications:

Reddy, B.G., Pohl, L.R. and Krishna, G.: The requirement of gut flora in nitro benzene-induced methemoglobinemia in rats. Biochem. Pharmacol. 22: 119-1122, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00832-02 LCP

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
A Simple Assay for Prostaglandin and Prostacycline Biosynthesis in the Lung

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

PI:	G. Krishna	Chief, Section on Drug-Tissue Interaction	LCP	NHLBI
	S. Giri	Guest Worker	LCP	NHLBI
OTHER:	N. Kim	Chemist	LCP	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Drug-Tissue Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland

TOTAL MANYEARS: 1.0	PROFESSIONAL: 1.0	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)

A simple method has been developed for the assay of enzymes involved in prostaglandin and prostacyclin synthesis. This involves incubation of various fractions from guinea pig lung homogenates with ^{14}C-arachidonic acid and cofactors, GSH and epinephrine. ^{3}H-Prostaglandins (E_1 and $F_{2\alpha}$) were included in the assay mixture to monitor the loss during the assay as well as during the isolation. Prostaglandins extracted with ethyl acetate and purified through a combination of column and thin layer chromatography were quantitated by measuring the radioactivity. $PGF_{2\alpha}$ and PGE_2 have been characterized by various techniques including reverse isotope dilution as well as radioimmunoassay. Prostacyclin formation was monitored by the formation of 6-keto $PGF_{1\alpha}$ mainly because of the extreme lability of the prostacyclin. A more direct assay of prostacyclin is being developed utilizing the ability of prostacyclin to specifically increase cyclic AMP in platelets.

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PHS-6040
(Rev. 10-76)

Project Description:

Objectives: It has been shown that lung is capable of synthesizing a number of prostaglandins from arachidonic acid. Since prostaglandins are known to be involved in various inflammations and since various compounds such as paraquat, ozone, etc. that damage the lung induce inflammatory response in the lung during the early phase of toxicity, it has been our objective to investigate the possibility that toxicants such as paraquat influence the prostaglandin metabolism in the lung. Since no reliable methods are available for the measurement of the enzymes involved in the synthesis of prostaglandins, we have developed a simple assay for the biosynthesis of prostaglandins in the lung. Recently, it has been shown in other systems such as coronary arteries and stomach that a new prostagladin, namely prostacyclin, is formed which appears to be more potent than other known prostaglandin in relaxing coronary arteries and inhibiting platelet aggregation. We have investigated the lung to see if it is also capable of synthesizing prostacyclin from arachidonic acid.

Methods Employed: Male Hartley guinea pigs (400-500 g) were used throughout the study. The animals were anaesthetized with pentobarbital and the lung perfused with cold saline. The lung was homogenized in Tris-HCl buffer (0.1 M pH 8.2). The homogenate was centrifuged at 600 x g to remove cell debri and the supernatant was used in the assay. In other experiments, various subfractions such as mitochondria, microsomes and soluble fractions were prepared and these fractions were employed for the assay. The enzymes were incubated with 10 µM of ^{14}C-arachidonic acid, 5 mM GSH and 5 mM of epinephrine in Tris-HCl buffer (pH 8.2, 0.1 M). ^{3}H-Prostaglandin (E_2 and $F_{2\alpha}$) (10 pmoles) were included in the assay to monitor the loss during the assay as well as during the isolation. The incubations were terminated at various time periods by addition of PGE_1 and PGE_2 and prostaglandins were extracted into 1.5 ml of acetyl acetate. The prostaglandins were separated from the substrate arachidonic acid by chromatography and silicic acid column [(18 ml of a mixture of toluene:ethyl acetate (60:40) quantitatively eluted arachiodonic acid from the column. All prostaglandins were then eluted with 2 ml of methanol)]. The prostagalndin fractions were evaporated to dryness under nitrogen and suspended in 50 µl of ethanol. Various aliquots were then rechromatographed either by TLC (Silica gel:developed with ethyl acetate:acetone:acetic acid - 90:10:1) or Silicic acid column chromatography [PGE_2 was eluted by 10 ml of a mixture of toluene:ethyl acetate (60:40); $PGF_{1\alpha}$ - 6 keto (a prostacyclin metabolite) and PGE_2 were eluted by 14 ml of a mixture of toluene, ethyl acetate and methanol (60:40:2); PGF_2 was eluted by 6 ml of a mixture of toluene, ethyl acetate and methanol (60:40:20)]. PGE_2 was separated from PGE_1 - 6 keto by conversion to PGB_2 by NaOH - PGE_1 - 6 keto was converted to a hydroxy compound by $NaBH_4$ - these were then separated by Silica acid chromatography as above. Radioimmunoassays were also performed on PGE_2 and $PGE_{2\alpha}$ fractions utilizing specific antiseria (in these experiments no labeled or unlabeled carriers were used). These fractions were also utilized for crystalization with authentic PGE_2 and $PGE_{2\alpha}$ to constant specific activity. Radioactivity in various fractions (both ^{14}C and

^3H) were determined and based on specific activity of arachidonic acid used in an assay, the rate of synthesis of prostaglandins were calculated. Based on the recovery of ^3H in the PGE_2 and $PGE_{2\alpha}$ fractions, it was possible to calculate the absolute amount of PGE_2 and $PGE_{2\alpha}$ in the assay.

Major Findings: The prostaglandin synthesis by guinea pig lung homogenate was linear with time (up to 15 min) and with protein concentrations (75-750 µg). Of the various fractions of the lung homogenate examined, microsomes had the highest activity for the synthesis of prostaglandins. The enzyme had an apparent affinity for the substrate of 30 µM and showed no apparent requirement for other cofactors. Identical results were obtained using either the TLC system or the column. Although the radioactivity associated with $PGF_{2\alpha}$ by reverse isotope dilution techniques, as well as by measurement by specific radioimmunoassay, only 30% of the radioactivity appears to be PGE_2 and the rest 60-70% to be $PGE_{1\alpha}$ - 6 keto as determined by covnersion of PGE_2 to PGB_2 and reduction of $PGF_{1\alpha}$ - 6 keto by $NaBH_4$. Moreover, a similar value is obtained by specific radioimmunoassay of PGE_2. (30 pmoles/mg protein/min of $PGF_{1\alpha}$ - 6 keto was formed by the lung enzyme). A more direct assay for prostacyclin formation has been developed. This assay utilizes the specific activation of adenylate cyclase and accumulation of cAMP in the platelets by prostacyclin. This assay has a potential sensitivity of detecting 1 pmole of prostacyclin.

Significance to Biomedical Research and to the Program of the Institute: With the advent of the development of a reliable and simple assay of enzymes involved in prostaglandin biosynthesis it should be possible to investigate the role of prostaglandins in both physiological and pathophysiological conditions. This is a useful tool to investigate whether drugs like paraquat have an effect on the synthetic machinary of prostaglandins, in order to explain the early inflammatory response in the lung induced by the drug. The important finding that the lung has a capacity to synthesize prostacyclin may have a greater impact in the understanding of the role of the lung in platelet hemostasis and thrombosis.

Proposed Course of Project: It is proposed to investigate the role of prostaglandins and prostacyclin in drug-induced lung damage. Since prostacyclin is synthesized in the lung, it is possible that the lung may be involved in maintaining platelets in a nonaggregated state. It is proposed that we study the role of drug-induced lung injury on the platelet function.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00834-02 LCP

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
The Role of Calcium and cyclic GMP in the Hormone-induced Lipolysis

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

PI:	R.M. Gaion	Visiting Fellow	LCP	NHLBI
	G. Krishna	Chief, Section on Drug-Tissue Interaction	LCP	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Drug-Tissue Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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)

Cyclic-AMP appears not to be the only modulator of the intracellular response to hormone stimulation in fat cells. A possible role for cyclic GMP and calcium flux at this level can be proposed. Epinephrine induced a very rapid efflux of calcium from fat cells within seconds and a marked increase in cGMP level which reached a peak within 30 sec. The increase of cAMP and the maximal stimulation of the lipolysis occurred at a later time (2-6 min). Theophylline did not stimulate calcium efflux nor had any early effect on cGMP, but stimulated the uptake of calcium, the accumulation of cGMP and cAMP and the rate of lipolysis 2-6 min. Calcium ionophore A 23187 stimulated calcium efflux and cGMP accumulation within seconds, did not affect cAMP but had a short stimulatory effect on lipolysis. Both dibutyryl cGMP and cGMP were able to mimic the effect of A 23187 on lipolysis. Calcium flux and cGMP formation could be the two very early steps of the intracellular response to hormone stimulation and could control the reaction chain that leads to the lipolytic response in fat cells.

Project Description:

Objectives: Changes in the lipolytic activity of fat cells have been generally shown to be related to parallel changes in cAMP level. But many findings cannot be explained solely on the basis of this model. The metabolic regulation of this process appears to be more complex. It has been shown by this laboratory and others that the concentration of cGMP was increased in fat cells by various agents, such as epinephrine, norepinephrine, acetylcholine, carbamylcholine, insulin and calcium ionophore A 23187. Some of these agents have also been shown to stimulate calcium flux. cGMP and calcium are already recognized to be two directly related factors in the control of the main metabolic functions in different kinds of cells. The main objective of this study is to estalbish whether the lipolytic response to hormone stimulation in fat cells is mediated or modulated by cGMP and to elucidate the role of calcium in this process.

Methods Employed: Isolated epididymal fat cells from Sprague-Dawley rats weighing 140-150 g were utilized for the study. cAMP and cGMP were converted to their succinyl derivatives which were measured by radioimmunoassay. Lipolysis was measured by the rate of glycerol release from fat cells in response to hormones. Calcium efflux and uptake were studied by isotopic measurements.

Major Findings: Calcium ionophore A 23187 (10^{-5}M) increased the cGMP level in fat cells within seconds. After attaining a peak at 30 sec, the nucleotide level fell to the basal value at 1 min and rose again at 2-6 min.

A 23187 (10^{-5}M) stimulated ^{45}Ca efflux from fat cells within seconds. This effect was more evident when EDTA (5 mM) was present in the incubation medium in order to chelate extracellular calcium and thus prevent its reuptake. A 23187 did not alter cAMP level, but increased the rate of lipolysis in fat cells during the first 6 min of incubation.

Epinephrine stimulated lipolysis by increasing cAMP. We have shown that it is also able to increase cGMP level and the time-response curve of this nucleotide was similar to that observed in the presence of A 23187 in that the concentration of cGMP reached a peak within 30 sec, then decreased to a lower level at 1 min, a second increase in cGMP occurred at a later time (2-3 min). Epinephrine had no effect on Ca^{++} uptake.

Theophylline (0.3 M) increased cAMP levels and lipolysis. It did not cause an early increase in cGMP accumulation as seen with other agents but induced an increase in cGMP at a later time (2-6 min). ^{45}Ca efflux from fat cells was not increased by theophylline, but the drug increased ^{45}Ca uptake.

cGMP (10^{-4}-10^{-3} M) and dibutyryl cGMP (10^{-4}-10^{-3}M) stimulated lipolysis for a short duration in a similar manner as the calcium ionophore, A 23187.

Insulin stimulated cGMP accumulation giving rise to a single peak within 2-6 min while acetylcholine, induced a rapid increase in the level of the nucleotide within seconds, followed by a later increase in cGMP level.

These data suggest that cGMP formation in fat cells may be mediated by calcium. Increases in cGMP occur when calcium efflux or uptake in the cells is increased, that is when the concentration of the ion in the cytoplasm undergoes a transient increase. This could be the signal for the stimulation of guanylate cyclase and thus an increased synthesis of cGMP.

cGMP appears also to play a role in the regulation of the lipolytic process. The nucleotide per se is able to stimulate lipolysis and it has been reported that this effect is mediated by an increase in cyclic AMP. These new findings would be useful in explaining the mechanism that control the adrenergic stimulation of fat cell function. The following model is proposed: 1) Interaction of the catecholamines with the receptor. 2) Depolarization of the cell membrane. 3) Stimulation of calcium efflux from the intracellular stores into the cytoplasm and from the cytoplasm into the extracellular space. 4) Stimulation of guanylate cyclase and formation of cGMP. 5) Activation of adenylate cyclase by the direct interaction of the hormone with the receptor coupled to adenylate cyclase. 6) Activation of lipolysis by cGMP and cAMP. Redistribution of calcium in the cell. Second stimulation of guanylate cyclase with the formation of the cGMP in order to maintain lipolysis.

Significance for Biomedical Research and the Program of the Institute: The new findings show a possible role of calcium and cGMP in the control of lipid mobilization from fat cells in response to adrenergic hormones. This could be a useful step for the understanding of some of the metabolic alterations involved in the pathogenesis of different types of hyperlipoproteinemias, as related to a lack of control of the lipolytic process in fat cells leading to adrenergic release of FFA into the blood stream. The hyposensitivity of fat cells to hormones due to some alterations in the metabolic steps that control lipolysis, has been recognized to represent an early step in the development of diabetes. A clear knowledge of the key mechanisms that control fat cell function is necessary for a more complete understanding of these pathological problems and a possible new pharmacological approach.

- Proposed Course of Project: The role of calcium, cGMP and other possible factors involved in the intracellular response to hormone stimulation in fat cells will be studied. From this point of view the cholinergic function in this system will also be examined. The changes induced in this system by altered metabolic states will be investigated.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00835-01 LCP

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
A Mechanism for the Metabolic Activation of Chloroform

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

PI:	L.R. Pohl	Senior Staff Fellow	LCP	NHLBI
	B. Bhooshan	Visiting Fellow	LCP	NHLBI
	G. Krishna	Chief, Section on Drug-Tissue Interaction	LCP	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Pharmacology

SE
Drug-Tissue Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	

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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)

When [^{14}C] $CHCl_3$ was incubated with liver microsomes in the presence of increasing concentrations of cysteine, the covalent binding of the [^{14}C] label to protein and the formation of [^{14}C] CO_2 where found to progressively decrease; concomitantly, the formation of [^{14}C] 2-oxothiazolidine-4-carboxylic acid increased. This compound, which is formed from phosgene, $COCl_2$, and cysteine, accounted for approximately 65% of the decreases in binding and liberation of CO_2. When [^{3}H] $CHCl_3$ was incubated with liver microsomes, no binding to protein was detected. These results establish that $CHCl_3$ is activated in vitro exclusively by metabolism of the C-H bond, and that metabolic oxidation of this bond to produce $COCl_2$ appears to be a major activation process. Base hydrolysis studies of microsomal protein containing covalently bound [^{14}C] label support this conclusion.

PHS-6040

Project Description:

Objectives: The liver and kidney toxicity produced by $CHCl_3$ has been shown by this laboratory to be mediated by a metabolic activation step, which is catalyzed by cytochrome P-450. Reactive metabolites appear to cause the toxicity since covalent binding of radiolabel to tissue appears to correlate with the degree of toxicity. However, the structure of these metabolites and the mechanism for their formation has not been elucidated until now.

We have reported in an accompanying study that $CHCl_3$ is metabolized to phosgene, $COCl_2$, by liver microsomes. This reactive compound, which is known to be toxic, would be expected to react covalently with tissue protein. In this investigation we have more clearly defined the mechanism of activation of $CHCl_3$. We report that $CHCl_3$ is activated exclusively by metabolism of the C-H-bond. Moreover, oxidation of this bond to $COCl_2$ appears to be a major route of activation of $CHCl_3$.

Methods Employed: [^{14}C] 2-oxothiazolidine-4-carboxylic acid was prepared from [^{14}C] cysteine and $COCl_2$ by a published method. Liver microsomes were prepared from male Sprague Dawley rats (170-200 g) which were pretreated for 3 days with phenobarbital (80 mg/kg,i.p.). In vitro incubations were conducted in sealed flasks employing [^{14}C] or [^{3}H] $CHCl_3$ as substrate, a NADPH-generating system and various concentrations of cysteine. The reactions were performed for 10 min and terminated by the addition of hydrochloric acid. The amount of covalent binding to microsomal protein was determined by a previously reported method. [^{14}C] CO_2 generated from each reaction was trapped in plastic wells containing 10 microliters of 8% sodium hydroxide absorbed onto a 1 cm x 1 cm piece of absorbant paper, and quantitated by scintillation spectrophotometry. Phosgene, $COCl_2$, produced from each reaction was trapped by cysteine as [^{14}C] 2-oxothiazolidine-4-carboxylic acid. After evaporation of the reaction mixture, this product was purified by anion exchange chromatography on a column containing 1 g of AG-1 x 8, 100-200 mesh resin, using an increasing concentration of formic acid as the eluent. The fraction eluting in 6 N formic acid was found by reverse isotope dilution to be virtually 100% 2-oxothiazolidine-4-carboxylic acid. This fraction was quantitated by scintillation spectrophotometry. The efficiency of recovery of [^{14}C] CO_2 and [^{14}C] 2-oxothiazolidine-4-carboxylic acid produced in each reaction was determined by performing the incubations with synthetic standards. These values were used to determine the absolute amounts of CO_2 and 2-oxothiazolidine-carboxylic acid produced in each reaction.

The chemical nature of the [^{14}C] material bound covalently to microsomal protein was studied by incubating the protein in 1N sodium hydroxide at room temperature for approximately 1 week. The resulting solution was then counted by scintillation spectrophotometry before and after acidification with hydrochloric acid.

Major Findings: When [^{14}C] $CHCl_3$ was incubated with liver microsomes in the presence of increasing concentrations cysteine, the covalent binding of the [^{14}C] label to protein and the formation of [^{14}C] CO_2 progressively decreased; concomitantly, the formation of [^{14}C] 2-oxothiazolidine-4-carboxylic acid increased (Table 1). This compound which is formed from phosgene, $COCl_2$, and cysteine accounted for approximately 65% of the decreases in binding and liberation of CO_2. When the incubations were conducted with [^{3}H] $CHCl_3$ no irreversibly binding to microsomal protein was found.

Hydrolysis of radiolabeled microsomal protein in sodium hydroxide, followed by acidification with hydrochloric acid resumed in approximately 50% loss of [^{14}C] label. This finding is consistent with at least 50% of bound radiolabel resulting from $COCl_2$ and forming -X-CO-X- linkages where X is sulfur, oxygen, or nitrogen. Hydrolysis of these bonds would yield CO_2 upon acidification and thus a loss of [^{14}C] label.

Significance to Biomedical Research and to the Program of the Institute: Chloroform which is widely used for industrial purposes, is toxic to the liver and kidney of man and experimental animals. Long-term chloroform administration produces liver tumors in mice. Recently several new studies have linked death rates due to bladder cancer and large intestinal cancer to the extent to which water is chlorinated. $CHCl_3$ is believed to be the carcinogen which is produced as a side product of the chlorination process. A clear understanding of how chloroform produces these toxicities will allow one to design safer environmental chemicals. The finding that chloroform is activated by an oxidative dechlorination process to produce phosgene is an important initial step in solving this problem. This discovery has practical importance, since it has defined a functional group containing a hydrogen and two or more halogens as a potentially reactive site, which should be avoided in the design of future drugs and environmental chemicals.

Proposed Course of Project: The results of this report indicate that $COCl_2$ is a major metabolite of $CHCl_3$ (Table 1). However, it appears that another route of activation also occurs, since $COCl_2$ does not account for all of the covalent binding to microsomal protein. This alternate route of metabolism also appears to involve metabolism of the C-H bonds, since [^{3}H] $HCCl_3$ did not bind irreversible to microsomal protein. The structure of this metabolite can likely be determined by isolating and purifying the amino acids to which it is bound. Identification of this structure by mass spectroscopy would permit the elucidation of the initial metabolite, and the metabolic step producing it.

Understanding the processes leading to the activation of $CHCl_3$ also has general application since many other compounds, such as the general anesthetic halothane and the antitumor agent o,p'-DDD contain similar functional groups. It is our intent to extend our studies to these and other therapeutically and environmentally important halocarbons.

Publication:

Sipes, G.L., Krishna, G. and Gillette, J.R.: Bioactivation of carbon tetrachloride, chloroform and bromotrichloromethane: Role of cytochrome P-450. Life Sci., 20: 1541-1548, 1977.

Table 1. Effect of trapping phosgene as 2-oxothiazolidine-4-carboxylic acid on the irreversible binding to microsomal protein and the production of CO_2 of [^{14}C] $CHCl_3$

Incubation conditions	Irreversible binding	CO_2	2-oxothiazolidine-4-carboxylic acid
	pmoles/mg protein/10 min		
No cysteine	2077	1684	0
0.5 M cysteine	1729	1404	810
1.0 M cysteine	1103	859	1229
2.0 M cysteine	593	775	1525
4.0 M cysteine	413	693	1872

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00836-01 LCP

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Identification of Phosgene as a Metabolite of Chloroform ($CHCl_3$)

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

PI:	L.R. Pohl	Senior Staff Fellow	LCP	NHLBI
	B. Bhooshan	Visting Fellow	LCP	NHLBI
	G. Krishna	Chief, Section on Drug-Tissue Interaction	LCP	NHLBI

COOPERATING UNITS (if any)
Mr. Noel Whittaker, Chemist, Laboratory of Chemistry, NIAMDD.

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Drug-Tissue Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.8	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

In this study we have characterized one of the reactive metabolites of $CHCl_3$ by incubating liver microsomes with [^{14}C] $CHCl_3$ in the presence of cysteine. This thiol reagent was found to block the covalent binding to microsomal protein and concomitantly trap a reactive metabolite of $CHCl_3$. The trapped product was identified as 2-oxothiazolidine-4-carboxylic by reverse isotope dilution and gas chromatography-mass spectrometry (GC-MS). When the incubation was repeated in an atmosphere of $^{18}O_2$, the trapped product contained virtually 100% $^{18}O_2$ in the 2-oxo position. These results are consistent with $CHCl_3$ being activated by cytochrome P-450 thru an oxidation of the C-H bond to produce $C(OH)Cl_3$ which would spontaneously dehydrochlorinate to yield phosgene, $COCl_2$. This reactive product, which is known to be toxic, could either react with microsomal protein or be trapped by cysteine to produce 2-oxothiazolidine-4-carboxylic acid. A similar oxidative dechlorination reaction leading to the formation of a reactive intermediate has previously been reported by this laboratory for the antibiotic, chloramphenicol.

Project Description:

Objectives: Previous studies from this laboratory have established that the kidney and liver toxicity produced by $CHCl_3$ is mediated by the formation of reactive metabolites, which bind irreversibly with tissue macromolecules. The activation of $CHCl_3$ is catalyzed by a cytochrome P-450 mixed-function oxygenase which is present in the microsomal fraction of the liver. To better understand the mechanism of this process we decided to attempt to trap a reactive metabolite of $CHCl_3$. Since it was previously found that the dichloromethyl carbon of chloramphenicol, R-$NHCOCHCl_2$, was oxidized by cytochrome P-450 of rat liver to a reactive intermediate which appeared to be R-NHCOCOCl, it was felt that a similar oxidative dechlorination process could occur in the activation of $CHCl_3$. If this were true, phosgene, $COCl_2$, would be formed. Since $COCl_2$ is a gas it would be difficult to isolate it unless it was chemically trapped. Cysteine was chosen as the trapping agent since it was previously established in the chemical literature that this thiol reagent reacts rapidly with $COCl_2$ to produce 2-oxothiazolidine-4-carboxylic acid.

Methods Employed: [^{14}C] $CHCl_3$ was obtained from New England Nuclear. $^{18}O_2$, 99% pure, was purchased from Stohler. 2-Oxothiazolidine-4-carboxylic acid was prepared from cysteine and phosgene by a reported procedure.

Male Sprague Dawley rats weighing 180-200 g were pretreated with phenobarbital for 3 days (80 mg/kg, ip) and their liver microsomes were isolated according to standard procedures.

In vitro incubations were conducted with liver microsomes, a NADPH-generating system, [^{14}C] $CHCl_3$ (1 mM) and in the presence or absence of cysteine (2 mM). Incubations were conducted for 10 min and terminated by the addition of methanol. Covalent binding of [^{14}C] $CHCl_3$ to the precipitated protein was determined according to previously published methods.

The methanol supernatants from the cysteine containing reactions were combined, evaporated and then dissolved in water. The aqueous solution (pH 6) was extracted with ethyl acetate and then acidified (pH 2) with hydrochloric acid. The acidic solution was extracted with ethyl acetate. The organic extract was then evaporated to yield a residue, which was characterized by reverse isotope dilution with 2-oxothiazolidine-4-carboxylic acid and GC-MS after derivatization with diazomethane.

The GC-MS were performed on a Finnigan 1015 D instrument which was equipped with a CI source, a Finnigan 9500 GC and a Model 6000 Data System. The samples were injected onto a glass column (2 m × 2 mm id) which was packed with 3% OV-225 on Gas Chrom Q, 100-120 mesh. Helium was employed as carrier gas at a flow rate of 25 ml/min. The injection port was operated at 225°. The mass spectrometer was operated at a source of 1 torr employing methane as the reagent gas. The source of temperature was 150°C, and the electron energy was 100 ev. GC-MS were also performed on the acidic extract of the mixture which was incubated in an atmosphere of $^{18}O_2$, and on an

authentic sample of methyl 2-oxothiazolidine-4-carboxylic, which was prepared from 2-oxothiazolidine-4-carboxylic acid and diazomethane.

Major Findings: Effect of cysteine on the in vitro covalent binding of of [^{14}C] $CHCl_3$. When [^{14}C] $CHCl_3$ was incubated 10 min with liver microsomes from phenobarbital pretreated rats, 2077 pmoles/mg protein of [^{14}C] label was found bound covalently to microsomal protein. The amount of covalent binding was decreased to 593 pmoles/mg protein when the incubations were performed in the presence of 2 mM cysteine. This observation indicated that cysteine deactivated a reactive metabolite of $CHCl_3$ which was subsequently binding irreversibly to microsomal protein.

Trapping phosgene as a reactive metabolite of $CHCl_3$. A radioactive fraction was isolated from the acidic extract of the incubation mixture of [^{14}C] $CHCl_3$ and 2 mM cysteine. Reverse isotope dilution analysis with authentic standard revealed that this fraction corresponded to 2-oxothiazolidine-4-carboxylic acid. This finding was confirmed after derivatizing the radioactive fraction with diazomethane and analyzing the product by methane GC-MS m/e (relative abundance), 162 (MH^+, 85), 134 (MH^+-CO, 50), 102 (MH^+-$HCOOCH_3$, 100) retention time of 6.69 min. This spectra and retention time were nearly identical to the authentic standard, methyl 2-oxothiazolidine-4-carboxylic acid.

The GC-MS of the product isolated from the reaction performed in an atmosphere of $^{18}O_2$ established that the 2-oxo oxygen was derived from molecular oxygen: m/e (relative abundance). 164 (MH^+-C^{18}O, 50), 104 (MH^+-$HCOOCH_3$, 100).

The reults clearly establish that phosgene, $COCl_2$,is a reactive metabolite of $CHCl_3$. This product results from $CHCl_3$ by an oxidative dechlorination reaction which likely involves the intermediacy of trichloromethanol (CCl_3OH) which would be expected to spontaneously dehydrochlorinate to yield $COCl_2$. This reactive metabolite could subsequently bind covalently to macromolecules or be trapped by cysteine to form 2-oxothiazolidine-4-carboxylic acid.

Significance to Biomedical Research and the Program of the Institute: The observation that phosgene is a metabolite of $CHCl_3$ is another example of a metabolic oxidation dehalogenation which produces a potentially toxic reactive metabolite. In last year's progress report, we reported the first documented example of such a bioactivation process in our studies with the antibiotic, chloramphenicol. This compound contains a dichlormethyl carbon which is hydroxylated to yield a reactive oxamyl chloride intermediate.

Other drugs and environmental chemicals also contain halosubstituted carbons. Many of these compounds, such as halothane (bromochlorotrifluoroethane) also produce a variety of toxicities. Therefore, it is possible that biotransformation by oxidative dehalogenation pathway has general importance in toxicology.

These mechanistic studies have an important practical application. By knowing the nature of the enzymatic process leading to a reactive toxic metabolite, it is possible to prevent toxicity by at least three general approaches:

1) Trap the toxic metabolite in vivo by the administration of a nontoxic agent which will react rapidly and specifically with the reactive metabolite to produce a product which is also safe and rapidly excreted.

2) Develop drugs which specifically inhibit the oxidative dehalogenation enzymes.

3) Design drugs and environmental chemicals which are not susceptible to activation by the oxidative dehalogenation pathway.

The oxidative dehalogenation process may also have an importance in pharmacology. For example, the halocarbon 1-(o-chlorophenyl)-1-(p-chlorophenyl)-2,2-dichloroethane (o,p'-DDD) has been shown to cause atrophy of the adrenal cortex. This specific toxicity has been employed to treat patients with adrenal cortical carcinoma. The mechanism for this action is unknown, but may involve an oxidative dechlorination process yielding a reactive acid chloride metabolite. It is this product which may be responsible for the activity of o,p'-DDD.

Proposed Course of Project: The study of the in vitro metabolism of $CHCl_3$ to phosgene is completed and a manuscript is in preparation. We next intend to determine if phosgene is an in vivo toxic metabolite of $CHCl_3$. This problem will be approached by blocking the toxicity of $CHCl_3$ with cysteine and attempting to trap phosgene as 2-oxothiazolidine-4-carboxylic acid. If this study is successful, we intend to extend our studies to other halocarbons such as the general anesthetic halothane, and the anti-tumor agent o,p'-DDD. Our ultimate goal is to use our knowledge of the mechanism of metabolic aetivation processes to design safer and more specific activating therapeutic agents.

Publication:

Nelson, S.D., Mitchell, J.R. and Pohl, L.R.: Application of chemical ionization MS and the twin-ion technique in the analysis of reactive intermediates in drug metabolism: In Frigerio, A. and Bhisalberti, E.L. (Eds.): Mass Spectrometry in Drug Metabolism. New York, Plenum Publishing Corporation, 1977, pp. 237-249.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00837-01 LCP

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
A New Synthesis of Radiolabeled Chloramphenicol and Stereoisomers

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

PI:	L.R. Pohl	Senior Staff Fellow	LCP	NHLCI
	G. Krishna	Chief, Section on Drug-Tissue Interaction	LCP	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Drug-Tissue Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	

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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 new procedure has been developed for the preparation of radiolabeled chloramphenicol (CAP), and its stereoisomers. This method involves the reduction of CAP ketone with $NaBH_4$ to quantitatively yield a 1:1 mixture of epimers, which are efficiently separated into the purified isomers by preparative HPLC. When $NaBH_4$ is used as the reducing agent, this procedure can be used to synthesize CAP and its stereoisomers of high specific activity.

Project Description:

Objectives: Our laboratory has ben studying the mechanism of bio-activation of chloramphenicol (CAP). To facilitate our studies we synthesized CAP with a [^{3}H] label in the benzylic position. This procedure is reported in a published paper and involves the reduction of CAP ketone with CaB^3H_4. Although this method is useful, it does not permit the synthesis of a product of high specific activity since the tritiated CAP has to be purified from the reaction mixture by reverse isotope dilution with nonradioactive CAP. We have developed a procedure which allows the reduced product to be purified directly by high pressure liquid chromatograph (HPLC). Furthermore, this method also permits the simultaneous synthesis of the epimer of CAP.

Methods Employed: CAP ketone was dissolved in absolute ethanol and reduced with $NaBH_4$. The reaction mixture was evaporated to dryness to yield an oil residue which was acidified with 3 N HCl and extracted with ethyl acetate. The organic extract was evaporated to dryness and the products were separated and purified by HPLC using a Spectra Physics/3500 chromatograph. The instrument was equipped with an ODS Spherisorb, 3 mm x 250 mm, 5 micron column and a Schoeffel variable wave length detector, which was adjusted to 278 nm. The separation was effected by using water as the eluant at a flow rate of 1 ml/min.

Major Findings: The reduction of CAP ketone with $NaBH_4$ yielded CAP and its epimer in approximately a 100% yield. The mixture was separated into the individual purified isomers by a single passage through the reverse phase column. The retention time of CAP was 20.0 min while its epimer was 15.5 min.

Significance to Biomedical Research and to the Program of the Institute: Studies on the mechanism of the antibacterial activity of CAP have indicated that this compound acts by blocking bacterial protein synthesis at the ribosomal level. It is believed that CAP binds in a specific manner with the ribosomes to elicit this activity. If CAP and its stereoisomers were available in high specific activity the nature of this binding process could be studied in greater detail. A clear understanding of this interaction could lead to the design of a more potent and specific acting antibiotic.

These same radiolabeled derivatives of CAP could also be used to further investigate the mechanism of aplastic anemia induced by CAP. For example, CAP of high specific activity could be employed in autoradiography studies to localize the site of binding and possibly toxicity of CAP in bone marrow cells.

Proposed Course of Project: The synthesis outlined in this report will be repeated with NaB^3H_4. The radioactive products will be used in our studies on the metabolism and toxicity of CAP.

Publication:

Pohl, L.R. and Krishna, G.: A study of the mechanism of the metabolic activation of chloramphenicol by rat liver microsomes. Biochem. Pharmacology, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00838-01 LCP

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Effect of the Gut Flora on the Metabolic Disposition of Warfarin

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

PI:	L.R. Pohl	Senior Staff Fellow	LCP	NHLBI
OTHER:	G.W. Elmer	Faculty Member		

COOPERATING UNITS (if any)

Dr. G.W. Elmer is a Faculty Member of the Department of Pharmaceutical Chemistry, University of Washington, Seattle, Washington.

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Drug Tissue Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

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)

The metabolic disposition of [^{14}C] warfarin was studied in germ-free and acclimatized germ-free rats. During 48 hr after the intraperitoneal administration, the germ-free rats excreted approximately a two-fold lower amount of the administered dose in the urine than the acclimatized rats. In contrast, the germ-free rats excreted approximately a two-fold larger amount of the administered dose in the feces than the acclimatized rats. These results indicate that the gut flora are involved in the metabolism and disposition of warfarin. They appear to affect the enterohepatic recycling of warfarin and its metabolites by hydrolyzing glucuronide and possibly sulfate conjugates.

Project Description:

Objectives: The therapeutically important anticoagulant warfarin is extensively metabolized in vitro and in vivo. Variation in level of the enzymes effecting these metabolic pathways is believed to be responsible for part of the inter and intra individual variability of anticogulation seen with warfarin.

Recently, it was found that warfarin and its metabolites are metabolized to polar conjugated products. The bile appears to be a major route of excretion for these metabolites. Since most of warfarin and its metabolites are excreted in the urine, these polar metabolites are believed to be hydrolyzed in the gut and reabsorbed. The intent of the present study is to determine the relative role of the gut flora in the hydrolysis and recycling of warfarin and its metabolites.

Methods Employed: Male germ-free rats were housed in special germ-free isolator. A group of germ-free rats were made nongerm-free by acclimatizing them in our normal animal room for approximately 2 weeks. Three germ-free and three acclimatized rats weighing approximately 350 g were administered [^{14}C] warfarin (2 mg/kg) by the intraperitoneal route. Urine and feces were collected every 12 hr for 48 hr. The amount of [^{14}C] label in the samples was determined by scintillation spectrophotometry.

Major Findings: During 48 hr after the intraperitoneal administration of [^{14}C] warfarin, the germ-free rats excreted approximately 15% of administered dose in the urine and 22% in the feces as radioactive warfarin and metabolites. In contrast, the acclimatized rats excreted approximately 24% in the urine and 14% in the feces.

Significance to Biomedical Research and the Program of the Institute: The intra and inter subject variability in the anticoagulation response to warfarin is a serious medical problem. This study indicates that the gut flora can significantly influence the metabolic disposition of warfarin. Since it is known that the composition of the gut flora can vary with diet, it is possible that diet may be another factor responsible for the variation in anticoagulation seen with warfarin.

Proposed Course of Project: The urine and feces samples from the germ-free and acclimatized rats are presently being analyzed for warfarin and its metabolites. A hplc assay was developed to rapidly and specifically measure these compounds. The results of this study should more clearly define the metabolic role of the gut bacteria in the disposition of warfarin.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00839-01 LCP

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Catecholamine-induced cGMP and cAMP Has Separate Role in Rat Pineal Gland

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

PI:	G. Krishna	Chief, Section on Drug-Tissue Interaction	LCP	NHLBI
	C.L. Kapoor	Visiting Fellow	LCP	NHLBI

COOPERATING UNITS (if any)

D.C. Klein is a Physiologist in the Behavioral Science Branch, NICHHD
M. Buda is a Visiting Fellow in the Behavioral Science Branch, NICHHD.

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Drug-Tissue Interaction

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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)

Various beta adrenergic agonists that increase the accumulation of cAMP are known to induce pineal N-acetyltransferase (NACT) activity. Even though beta adrenergic receptors control both cAMP and cGMP accumulation, cGMP has no apprent role in the increase of NACT. Histochemical localization of cyclic nucleotide indicates that even though most of the cGMP is localized in the nerve innervating the gland it also appears to be present in pinealocytes even in denervated glands and catecholamines are markedly decreased. It is possible that cGMP may play a role in the catecholamine and serotonin secretion from the nerve ending in the pineal gland. Studies in isolated pinealocytes from newborn rats indicate that only cAMP is increased 100-200 fold by various catecholamines which is associated with the induction of NACT. cAMP appears to be necessary for maintaining the activity of NACT since lowering of cAMP causes an immediate lowering of the enzyme activity in the pinealocyte and is reversed by dibutyryl cAMP.

Project Description:

Objectives: The activity of N-acetyltransferase (NACT) is markedly increased by catecholamines (norepinephrine, epinephrine and isoproterenol) in the pineal gland. cGMP has also been shown to be increased by catecholamines. We have investigated the possibility whether beta adrenergic control of cGMP and cAMP has any role in either the induction or in the maintenance of elevated activity of NACT. Since catecholamines do not increase cGMP in dispersed cultured pinealocytes, we have used this preparation to study the effects of catecholamines on cAMP accumulation and induction of NACT activity and to determine whther beta adrenergic blocking agents cause an abrupt decrease in both cAMP levels and NACT activity.

Methods Employed: Intact and surgically denervated rat pineal glands were cultured in petri dishes containing BGJb Fitton Jackson media as described by Kleine and Weller (J. Pharm. Exp. Therap. 86:516,1973). In each dish 4 to 6 pineal glands were incubated with indicated concentrations of the various agents at 37°C under gas phase of 95% O_2-5% CO_2. Dispersed pinealocytes were cultured according to the procedure of Buda and Klein (Trans. of the Am. Soc. for Neurochem. 8:221, 1977). The cAMP and cGMP present in the cultured glands and cell pellets were extracted by sonication of the glands and cells in 0.5 ml of 5% trichloroacetic acid. The extract was centrifuged at 8,000 x g for 20 min. The precipitate was dissolved in 1 N NaOH and a portion was used for determination of protein; bovine serum albumin was used as standard (Lowry et al., J. Biol. Chem. 193:265,1951).

Cyclic nucleotides were assayed by radioimmunoassay procedure of Frandsen and Krishna (Life Sciences 18:529,1976). Samples containing low levels of cyclic nucleotides were assayed after succinylation.

Major Findings: 1) We have studied the effects of various catecholamines on cGMP and cAMP accumulation and on the activity of N-acetyltransferase (NACT) in intact and surgically denervated rat pineal glands in vitro culture. With glands from normal animals norepinephrine (NE) (10μM), epinephrine (E) (10μM) and isoproterenol (ISO) (1μM) induced 20-40 fold increase in both cAMP and cGMP at 5 min, followed by 20-40 fold increase in NACT at 4 hr.

2) These agents caused an immediate accumulation of cAMP and cGMP followed by an induction of NACT over a wide range of concentrations (1 nM-100 μM). (ED50 for NE 10^{-6}M, E 10^{-6}M and ISO 10^{-7}M). The relative potency of the l isomers of agonists were about 1000-fold more active than d isomers.

3) The surgical denervation of pineal gland resulted in a 2-3 fold increase of the maximal accumulation of cAMP and NACT activity caused by the catecholamines but a 70-80% decrease in accumulation of cGMP; l-propranolol inhibited the catecholamine induced cAMP and cGMP accumulation. The concentrations required to inhibit the accumulation of cAMP and cGMP were similar (0.5μM). Even though the adrenergic receptors controlling cAMP and

cGMP accumulation appear to be similar. These results suggest that a temporal correlation exists between cAMP accumulation and the induction of NACT while apparently no correlation exist between cGMP accumulation and enzyme induction.

4) Histochemical localization of cyclic nucleotides in pineal gland indicate that even though cGMP appears to be mostly localized in the innervating gland, appreciable amounts of cAMP was found in the denervated gland which was markedly increased by catecholamine stimulation. It is possible that cGMP may play a role in the secretion of catecholamine or serotonin from the pineal gland; if cGMP may have a role in the pinealocyte function remains to be elucidated.

5) Dispersed pinealocytes have been used to study the role of cAMP in the maintenance of N-acetyl transferase activity which has been induced 100-fold by incubating the cells with NE (1μM) for 3 hr.

When l-propranolol was added to the medium, it caused an abrupt reduction (90% in 5 min)in the intracellular level of cAMP followed by a rapid decline in NACT activity (70% at 15 min). This decrease was not induced by d-isomer of propranolol.

6) In pinealocytes which contain barely detectable levels of cGMP, there was little or no change in the cGMP concentrations either during the time course of cAMP accumulation and enzyme induction caused by NE. This together with the observation that the inhibitory effect of l-propranolol on NACT was reversed by dibutyryl cAMP and phosphodiesterase inhibitors such as theophylline or 3-isobutyl-methyl xanthine (IBMX) indicate an abrupt decrease in cAMP may be the signal for rapid decrease in pineal N-acetyltransferase activity and cAMP is involved in maintenance of NACT activity.

Significance to Biomedical Research and the Program of the Institute: The finding that cAMP is involved in the stimulation and in the maintenance of pineal N-acetyltransferase should greatly help in the understanding of the role of cyclic nucleotides in other systems when cAMP has been shown to induce certain enzymes such as tyrosine hydroxylase in sympathetic neurons and adrenal medulla, where it may be involved in the maintenance of the enzyme activity.

Proposed Course of Project: The mechanism of "turn-off" of pineal NACT preceeded by a rapid decrease of cAMP will be investigated in detail. If cGMP plays a role in the release of catecholamine and serotonin from pineal gland it will also be investigated.

Publications:

Klein, D.C., Buda, M.J., Kapoor, C.L. and Krishna, G.: Pineal serotonin N-acetyltransferase activity: An abrupt decrease in cyclic AMP may be the signal for "turn-off". Science, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE OFFICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00840-01 LCP

PERIOD COVERED
July 1, 1976 to September 30 1977

TITLE OF PROJECT (80 characters or less)

Studies on Chemical Modification and Metabolism of Diphenylhydantoin

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

PI:	B. Bhooshan	Visiting Fellow	LCP	NHLBI
	G. Krishna	Chief, Section on Drug-Tissue Interaction	LCP	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Drug-Tissue Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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)

A number of diphenylhydantoin (DPH) derivatives were synthesized and their anticonvulsant properties were evaluated by the maximal Electroshock Seizure test described by Swinyard et al., in 1952. Substitution of one of the phenyl rings of DPH with halogen (4-Cl or F) or substitution of all the halogens in one or both of the phenyl rings (d_5 or d_{10}) did not markedly alter the Ed50 values. However, substitution of both the phenyl rings with halogen (4,4'-Cl,Cl or F,F) markedly altered the ED50 values. Results obtained in this study indicate that substitution of the phenyl rings of DPH prolongs therapeutic efficacy and decreases acute neurological toxic effects.

Project Description:

Objectives: Diphenylhydantoin (DPH) is one of the most effective drugs available for the control of epilepsy. However, its usefulness is limited by a severe side effect namely gingival hyperplasia which affects 50% of the children who are treated. It is possible that the drug itself causes gingival hyperplasia or the toxicity is probably related to the metabolism of DPH. Since DPH is mainly metabolized by hydroxylation of the phenyl rings possibly involving epoxidation pathway, it is conceivable that the highly reactive metabolite may be involved in the toxicity. It may also bind covalently to tissue macromolecules resulting in the toxicity. In order to test these hypotheses, various analogues of DPH were synthesized and their effectiveness as anticonvulsants was tested and compared for their capacity in producing gingival hyperplasia.

Anticonvulsant activity: All the compounds synthesized were evaluated for their antiepileptic activity by maximal Electroshock Seizure test according to the method described by Swinyard et al., J. Pharmacol. Exp. Ther. 106:319 (1952). Maximal electroshock seizures are elicited with a $60H_z$ alternating current of 50 mA delivered for 0.2 sec via corneal electrodes. In all normal mice this stimulus will produce a maximal seizure which typically consists of a short period of initial tonic flexion and a prolonged period of hind limb tonic extension, followed by terminal clonus. Abolition of the hind limb tonic extension component of the seizure, 0.5 hr and 4 hr after the i.p. injection of a test drug dissolved in 30% PEG 400, is defined as a protection and indicates anticonvulsant activity in the test compound. Male mice weighing 25 g (N.I.H. all purpose strain) were used in these studies. Table 1 shows the data obtained.

In vivo covalent binding:- Sprague Dawley rats weighing 150-180 g, were injected with phenobarbitone sodium at 80 mg/kg for 3 days. On the 4th day, the livers were removed and microsomes were prepared. DPH [^{14}C] was incubated (purified by TLC on Silica gel) with microsomes and NADPH and its generating system and the covalent binding to microsomal protein was determined by methods previously described.

Major Findings: Substitution of one of the phenyl rings in DPH (4-chloro or fluoro) or substitution of all the hydrogens by deuterium in one or both of the rings altered only 2-3 fold of the ED50values (Table 1). However, substitution of both the rings with halogens (4,4'-Cl, or F) markedly altered the ED50 values as well as the time required to attain a peak response. The difluoro derivative was effective in mice for 10 days after a single dose of 2,000 mg/kg apparently without eliciting any neurological symptoms. A similar dose of DPH kills all animals. These results indicate that substitution of the phenyl rings of DPH prolongs therapeutic effects and it may lower the acute neurological toxicity induced by DPH.

Significane to Biomedical Research and Program of the Institute: The finding that it is possible to modify the drug DPH without significantly altering its efficacy as an anticonvulsant should greatly help in the study of the mechanism of gingival hyperplasia produced by DPH.

Proposed Course of Project: DPH has been shown to induce gingival hyperplasia in ferrets. The possibility of these DPH derivatives in inducing hyperlasia in ferrets is being attempted. It is also proposed to correlate the serum levels of the drugs with their anticonvulsant activity.

Publicatons: None

Table 1

DPH Derivatives	Time hr	ED50 mg/kg,i.p.
DPH	4	5
p-fluoro phenyl	4	15
p-chloro phenyl	4	15
p,p'-difluorodiphenyl	24	250
p,p'-dichlordiphenyl	24	250
pentadeuterophenyl	4	10
decadeuterodiphenyl	4	10

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00841-01 LCP

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Studies on the mechanism of metabolic activation of chloramphenicol

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

PI:	G. Krishna	Chief, Section on Drug-Tissue Interaction	LCP	NHLBI
	B. Bhooshan	Visiting Fellow	LCP	NHLBI
	L. Pohl	Senior Staff Fellow	LCP	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
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NHLBI, NIH, Bethesda, Maryland 20014

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)

Chloramphenicol is a well known broad spectrum antibiotic. Unfortunately it produces aplastic anemia in one out of 20,000 people who are treated. This laboratory has reported earlier that chloramphenicol is metabolically activated to some "reactive intermediate(s)" which bind covalently to tissue macromolecules followed by alteration in cellular integraty and probably cell death. In order to understand the mechanism of the formation of reactive intermediate(s) of chloramphenicol protein containing covalently bound chloramphenicol [^{14}C or ^{3}H] was hydrolyzed with Pronase and the hydrolyzates analyzed in an Aminco Acid Analyzer. The results obtained so far indicate that most likely bioactivated chloramphenicol binds covalently to four amino acids in the protein of liver microsomes.

Project Description:

Objectives: Chloramphenicol is a very useful broad spectrum antibiotic; however, its usefulness is limited due to its ability to produce aplastic anemia in certain sensitive individuals. This laboratory has reported earlier that chloramphenicol is activated (in phenobarbitone sodium pretreated rats) to chemically reactive intermediates which bind covalently to different tissue macromolecules. It was speculated at that time that metabolic activation of chloramphenicol to reactive intermediates (and resultant covalent binding to macromolecules) may be related to its ability to induce aplastic anemia. The main objective of this study has been to understand the biochemical mechanism by which 1) chloramphenicol is metabolically activated to chemically reactive metabolites and 2) binds covalently to various tissue macromolecules.

Methods Employed: Male Sprague Dawley rats, weighing 150 to 180 g were pretreated with phenobarbitone sodium at 80 mg/kg for 3 days. On the 4th day, livers were removed and microsomes were prepared by centrifugation at 100,000 g for 2 hr. Both, chloramphenicol labeled at benzylic carbon [^{3}H] and another at dichloroacetyl side chain [^{14}C] were used in the experiment. The enzyme preparation (microsomes) was incubated with chloramphenicol [^{3}H] or [^{14}C] for 10 min and the chloramphenicol bound protein was precipitated (with 10% TCA) and washed 10 times with methanol:ether (3:1). This protein was hydrolyzed by incubation with Pronase at 37° for 3 days, and the hydrolyzates, containing amino acids and amino acids-bound chloramphenicol, were analyzed on Perkin-Elmer KLA-3B Amino Acid Analyzer.

Ninhydrin was replaced with distilled water and was allowed to drain (rather than going into the reaction coil). The reaction coil was ketp at room temperature (instead of 115°) and the fractions were collected each minute from the "waste" end using LKB fraction collector. Each fraction was counted for radioactivity [^{3}H or ^{14}C].

Major Findings: Both chloramphenicol [^{3}H] and chloramphenicol [^{14}C] were found to bind covalently to rat liver microsomal proteins to almost the same extent. Amino acid analysis of the protein hydrolyzate indicated that about 40% of its radioactivity accounted for chloramphenicol metabolites covalently bound to amino acids. Protein hydrolyzate obtained from chloramphenicol [^{3}H] showed 4 bands - one in the basic and aromatic amino acid region and three in the acidic and neutral amino acid region. On the other hand, chloramphenicol [^{14}C] showed only three bands as shown in Table 1. This discrepancy is difficult to explain but it is possible that the metabolic activation occurs at the aromatic ring (at the nitro group) followed by covalent binding and hydrolysis of the dichloroacetyl side chain during isolation procedures.

One might speculate that the chemically reactive intermediate(s) of chloramphenicol, probably the oxalyl chloride, couples with histidine, lysine or cysteine of the tissue macromolecules. This can be confirmed by

chemical synthesis of these amino acid coupled chloramphenicol derivatives. Work in this direction is in progress.

Significance to Biomedical Research and Program of the Institute: A clear understanding of the mecchanism of metabolic activation of chloramphenicol to different reactive intermediates will help medicinal chemists in the design and synthesis of a chloramphenicol derivative which will be more potent than chloramphenicol as a broad spectrum antibiotic, but would be devoid of the dangerous toxic effect of this drug - namely aplastic anemia.

Proposed Course of Project: Isolation and identification of the chloramphenicol bound amino acids from the tissue macromolecules by synthesis of amino acid derivatives with chloramphenicol metabolite will be attempted.

Publications: None

Table 1

Band	Basic and aromatic amino acids	Acidic and Neutral amino acids		
	I	II	III	IV
	29 min	72 min	83 min	125 min
Relative percentage of the peak [^{3}H]	32	13	14	41
Relative percentage of the peak [^{14}C]	63	27	10	-

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE OFFICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZC1 HL 00851-04 LCP

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Paraquat Toxicity in Rat and Mouse

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

PI:	H.M. Maling	Chief, Section on Physiology	LCP	NHLBI
OTHERS:	E.A.B. Brown	Pharmacologist	HE	NHLBI
	J.R. Gillette	Chief, Lab. of Chem. Pharm.	LCP	NHLBI
	W. Saul	Chemist	LCP	NHLBI

COOPERATING UNITS (if any)

Hypertension-Endocrine Branch, NHLBI

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Section on Physiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 1.0	PROFESSIONAL: 0.5	OTHER: 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)

This project is a study of mechanisms involved in the pulmonary toxicity of paraquat, an herbicide used in agriculture throughout the world. The potentiation by l-isoproterenol of paraquat lethality (Ann.Rep. for 1975-76) can be explained by a decreased glomerular filtration rate and a reduced renal plasma flow: l-isoproterenol reduced the "average body clearance" of inulin and both acidic (p-aminohippuric acid) and basic (paraquat and N-methylnicotinamide) compounds. Nonreversible binding of ^{3}H-DL-epinephrine (5×10^{-4}M) to rat lung microsomes in vitro was increased about 30% by paraquat (5×10^{-3}M), was partially blocked by either superoxide dismutase (100 µg/ml, 63%) or catalase (30 µg/ml, 20-30%), and more completely blocked (70-86%) by the combination of superoxide dismutase and catalase. Nonreversible binding of epinephrine to lung microsomes was inhibited almost completely by glutathione (10^{-3}M, 96%) or ascorbic acid (10^{-3}M, 89%). However, extrapolation of these in vitro findings to paraquat toxicity in vivo is not justified since nonreversible binding of ^{3}H-DL-epinephrine did not occur in incubations with lung slices or homogenates.

Project Description:

Objectives: During 1976-1977, experiments have been performed with the following objectives: 1) to determine the mechanisms involved in the reduction by l-isoproterenol of paraquat "average body clearance", 2) to detect the effect of paraquat on the formation of superoxide anions and peroxides by measuring the nonreversible binding of epinephrine and other catechols to microsomal protein.

Methods Employed: The "average body clearance" of inulin, which was assumed to represent the glomerular filtration rate, was calculated from the relationship CL = Dose/AUC, in which AUC was the area under the curve of the plasma disappearance of ^{14}C-labeled inulin. Nonreversible binding to lung microsomes of ^{3}H-epinephrine was measured after incubation for 10 min of the labeled catecholamine in phosphate buffer containing a NADPH-generating system, lung microsomes (2 mg protein/ml), and appropriate additions such as paraquat (5×10^{-3}M).

Major Findings: Isoproterenol (0.3 mg base/kg, s.c.) reduced the clearance of inulin by about 50%, as calculated from the plasma disappearance curve; this corresponds to a 50% reduction in the average glomerular filtration rate. In our report for 1975-1976, we stated that l-isoproterenol reduced the clearance of both basic (paraquat and N-methylnicotinamide) and acidic (p-aminohippuric acid) compounds. We therefore concluded that it probably reduced renal blood flow. The reductions in clearances of inulin, p-aminohippuric acid, and N-methylnicotinamide indicate that a reduced glomerular filtration rate and a decreased renal blood flow both contributed to the decreased clearance of paraquat.

Table 1 shows the effect of various additions to the incubation mixture on the nonreversible binding of labeled epinephrine to lung microsomal protein. Paraquat (5×10^{-3}M) increased the nonreversible binding of epinephrine to lung microsomal protein by about 30% (Table 1,A,B). This binding required superoxide anion, since it was reduced more than 60% by superoxide dismutase (C,D, Table 1). Peroxides were also implicated since catalase reduced the binding by 20-30% (E,F). More complete inhibition of epinephrine binding was achieved by the combination of superoxide dismutase and catalase (G,H). However, one cannot conclude that paraquat produces its toxicity by stimulating the production in vivo of superoxide anions and peroxides since nonreversible binding of epinephrine could not be demonstrated in lung slices and homogenates.

Significance to Biomedical Research and the Program of the Institute: The experiments on isoproterenol-induced potentiation of paraquat toxicity involve a mechanism of toxicity which is usually ignored. This mechanism cannot be demonstrated in vitro. A reduction in glomerular filtration rate and renal plasma flow should affect the toxicity of many compounds which are not metabolized and which are rapidly excreted by the kidneys.

Proposed Course of Project: We plan to study the effects of l-isoproterenol on the toxicity and body clearance of various drugs. Additional efforts will be made to detect the stimulation by paraquat of the production of superoxide anions by lung slices.

Publications:

Maling, H.M., Saul, W., Williams, M.A., Brown, E.A.B. and Gillette, J.R.: On the mechanism of the potentiation by beta adrenergic agonists of paraquat toxicity in rats and mice. In Autor, Ann (ed.): Biochemical Mechanisms of Paraquat Toxicity. New York, Academic Press, 1977, in press.

Maling, H.M., Saul, W., Williams, M.A., Brown, E.A.B. and Gillette, J.R.: Reduced body clearance as the major mechanism of the potentiation by beta-2 adrenergic agonists of paraquat lethality in rats. Toxicol. Appl. Pharmacol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00852-01 LCP

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Isoproterenol on "Average Total Body Clearance" of Various Compounds in Rats

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

PI:	H.M. Maling	Chief, Section on Physiology	LCP	NHLBI
OTHERS:	W. Saul	Chemist	LCP	NHLBI
	W.J. Yasaka	Guest Worker	LCP	NHLBI
	J.R. Gillette	Chief, Lab. of Chem. Pharm.	LCP	NHLBI
	M.C. Wu	Statistician	HV BR	NHLBI
	J.H. Ware	Statistician	HV BR	NHLBI

COOPERATING UNITS (if any)
Biometrics Research Branch, Division of Heart and Vascular Diseases, NHLBI.

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Section on Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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)

In this project, we test the hypothesis that treatment with l-isoproterenol may enhance toxicity by decreasing the "average total body clearance" of many compounds, including drugs, that are not metabolized significantly and are excreted rapidly and almost entirely in urine. l-Isoproterenol (0.3 mg base/kg, s.c. administered within 10 sec of the i.v. injection of a labeled compound) reduced by 30 to 70% the "average total body clearance" of the following ^{14}C-labeled compounds: inulin, p-aminohippuric acid, N-methylnicotinamide, tetraethylammonium bromide, decamethonium bromide, hexamethonium dichloride, and penicillin. Salbutamol also decreased the "average total body clearance" of hexamethonium by about 70%. Treatment with l-isoproterenol decreased by 40 to 66% the LD50 values for pentamethonium, hexamethonium, decamethonium, and tetramethylammonium and by 20% that of tetraethylammonium. These effects can be explained by an apparent decrease in the glomerular filtration rate and a reduced renal plasma flow or by a reversible decrease in the number of functioning nephrons.

Project Description:

Objectives: The vascular actions of l-isoproterenol may decrease the "average total body clearance" and LD50 of a compound which is not metabolized appreciably and which is excreted rapidly and almost entirely in urine. In this project, we are studying this type of drug interaction, which cannot be demonstrated in vitro.

Methods Employed: "Average total body clearance" is determined as Dose/AUC, in which AUC is the area under the plasma disappearance curve. Plasma levels of radioactivity are measured in blood samples obtained from the retro-orbital sinus at appropriate intervals after injection in a rat's tail vein of a ^{14}C-labeled compound.

Major Findings: Table 1 shows the effect of treatment with l-isoproterenol (0.3 mg base/kg, s.c. within 10 sec of the i.v. injection of a ^{14}C-labeled compound) on the "average total body clearance" of various compounds. Since l-isoproterenol reduced the "average total body clearances" of inulin and both acidic and basic substances, isoproterenol-induced increases in toxicity of various compounds (Table 2) can be explained by a decrease in their clearance. Isoproterenol treatment does not affect the clearance of a substance like 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) which is excreted slowly, even though this substance is not metabolized and is excreted almost entirely in urine.

The β-2 adrenergic agonist, salbutamol, reduced the "average total body clearance" of hexamethonium by 70%. Salbutamol also reduced the LD50 of hexamethonium in rats by 44.8%. These findings suggest that the reduction in clearance of various compounds by l-isoproterenol and the associated increase in toxicity are β-2 adrenergic effects.

The LD50 of hexamethonium in mice was reduced 41.2% by isoproterenol treatment. We have previously reported (Pharmacologist 18:244, 1976) that isoproterenol potentiates paraquat toxicity in mice and that this potentiation is blocked by pretreatment with β-adrenergic blocking agent, dl-propranolol. These findings support the hypothesis that isoproterenol-induced decreases in "average total body clearance" and LD50 values for various compounds are β-adrenergid efffects and that these effects can be demonstrated both in mice and rats.

Significance to Biomedical Research and the Program of the Institute: Isoproterenol, salbutamol and β-2 adrenergic agonists are valuable in the treatment of asthma because they dilate the bronchi. Physicians should be aware of the possible reduction by these compounds of the "average total body clearance" and DD50 values of various other compounds which are not metabolized appreciably and which are excreted rapidly and almost entirely in urine.

Proposed Course of Project: We are puzzled by the relatively low value we obtained for the body clearance of p-aminohippuric acid. We expected to obtain our highest value of clearance for this compound, since its clearance is widely used as a measure of renal plasma flow. We are planning to measure the clearance of p-aminohippuric acid at 4 or 5 different doses, in order to establish the relationship between dose and "average total body clearance", when measured by the method used in this project.

Publications: None

Table 1. Effect of treatment with l-isoproterenol (0.3 mg base/kg, s.c. within 10 sec. of i.v. injection of ^{14}C-labeled compound) on "average total body clearance" of various compounds.

Compound	Average total body clearance: Saline s.c.	Average total body clearance: l-Isoproterenol s.c.	% Decrease
	ml/kg/min		
Inulin	7.24 ± 0.56 (4)	3.46 ± 0.20 (4)	52
p-Aminohippuric acid	13.19 ± 0.98 (6)	7.58 ± 0.43 (6)	43
Penicillin G	9.42 ± 1.69 (4)	5.04 ± 0.49 (4)	46
2,4,5-Trichlorophenoxy-acetic acid	0.47 ± 0.04 (4)	0.46 ± 0.40 (4)	2
N-Methylnicotinamide	9.91 ± 0.93 (6)	6.78 ± 0.86 (5)	32
Paraquat	8.39 ± 0.54 (4)	4.44 ± 0.23 (4)	47
Tetraethylammonium bromide	25.05 ± 2.05 (7)	15.08 ± 1.75 (7)	40
Decamethonium	5.31 ± 0.79 (6)	3.32 ± 0.33 (6)	37
Hexamethonium	7.72 ± 0.46 (4)	2.38 ± 0.25 (4)	69

Table 2. Intraperitoneal LD50 values for various compounds in male Sprague-Dawley rats (150-200 g) treated with saline or l-isoproterenol (0.3 mg base/kg, s.c.)

Compound i.p.	Saline s.c.	l-Isoproterenol s.c.	% Decrease
	mg/kg, i.p. (95% confidence limits)		
Paraquat	22.3 (21.6,22.9)	10.0 (8.0,12.7)	55.2
Pentamethonium bromide	177.8 (146.2,216.2)	59.7 (50.0, 71.4)	66.5
Hexamethonium dichloride	92.0 (79.6,106.4)	34.1 (29.8,39.2)	62.9
Decamethonium bromide	4.7 (3.9, 5.7)	2.4 (1.9,2.9)	49.9
Tetramethylammonium bromide	24.6 (20.3,30.0)	12.9 (10.6,15.6)	47.8
Tetraethylammonium bromide	108.0 (95.6,118.3)	86.6 (79.0,94.9)	19.8
Tolazoline hydrochloride	147.3 (124.3,174.5)	116.4 (98.1,138.0)	NS
Bretylium Tosylate	65.9 (52.0,62.3)	51.0 (46.6,55.8)	NS

Project No. Z01 HL 00852-01 LCP

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00853-01 LCP

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Modification of Drug Action by Liver Microsomal Enzyme Induction with Glycofurol

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

PI:	W.J. Yasaka	Guest Worker	LCP	NHLBI
OTHER:	H.A. Sasame	Chemist	LCP	NHLBI
	H.M. Maling	Chief, Section on Physiology	LCP	NHLBI
	J.R. Gillette	Chief, Lab. of Chem. Pharm.	LCP	NHLBI

COOPERATING UNITS (if any)

Dr. Yasaka is supported by Fundacao de Amparo a Presquisa do Estado de Sao Paulo, Brazil and Hoffmann-La Roche.

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Section on Physiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

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)

Glycofurol (tetraglycol) has been used in several countries as a solvent for drugs administered parenterally, although it has not been cleared for use in the U.S.A.. When administered i.p. (1 ml/kg 2X/day for 4 days) to male Sprague-Dawley rats (150-200 g), glycofurol increased significantly in liver microsomes the concentrations of cytochrome P-450 (65%), cytochrome b_5 (34%), NADPH-cytochrome c reductase (86%), and the activities of ethylmorphine demethylase (51%) and benzopyrene hydroxylase (74%). Glycofurol treatment decreased hexobarbital sleeping time (60%). Upon awakening, plasma and brain levels of hexobarbital were slightly (<22%) lower in treated than in control rats. A reduction (30%) in zoxazolamine paralysis time was correlated with an increase (38%) in liver microsomal metabolism and substantial decreases in plasma (55%) and brain (37%) zoxazolamine levels upon recovery of the righting reflex. We conclude that glycofurol treatment induees hepatic microsomal enzymes.

281

PHS-6040

Project Description:

Objectives: Glycofurol is a suitable injectable solvent for benzodiazepines, antibiotics, vitamins, and other drugs with high oil-water partition coefficients. Although a solvent is usually considered to be inert, it may modify the pharmacologic actions of the drug dissolved in it. The objectives of this project are to study the effects of the injectable solvent, glycofurol 75, on hepatic drug metabolism and on the pharmacologic action of drugs, like hexobarbital and zoxazolamine, which are metabolized by hepatic microsomes.

Methods Employed: Standard biochemical procedures were used to measure cytochrome P-450, cytochrome b_5, NADPH cytochrome c reductase, ethylmorphine demethylase, benzopyrene hydroxylase, and other parameters.

Major Findings: A single large dose (3.75 ml/kg, i.p.) of glycofurol did not affect significantly the hepatic microsomal cytochrome P-450 system, as evaluated by measurements at 3,6 and 12 hr of cytochrome P-450 and *in vitro* metabolism by hepatic microsomes of hexobarbital and zoxazolamine. However, hexobarbital sleeping times and zoxazolamine paralysis times were prolonged when glycofurol was used as a solvent in concentrations ranging from 12.5 to 50% in experiments with zoxazolamine and 12.5 to 100% in experiments with hexobarbital; the injection volume was kept constant at 5 ml/kg. All rats which received 50 mg/kg zoxazolamine in 50% glycofurol died within 30 to 90 sec. Hexobarbital sleeping time was unchanged when 12.5% glycofurol was the solvent. With higher concentrations of glycofurol, hexobarbital sleeping time increased about two-fold with 50% glycofurol as the solvent and about five-fold with 100% glycofurol. Similar effects were seen with zoxazolamine, with a significant increase in paralysis time even at the lowest concentration of glycofurol.

Repeated moderate doses (1 ml/kg 2X/day for 4 days) of glycofurol induced hepatic microsomal enzymes. This conclusion is based upon measurements made 13-18 hr after the last dose of glycofurol in rats injected 2X/day for 4 days. Some effects of this treatment are listed in Table 1.

Significance to Biomedical Research and the Program of the Institute: A solvent is usually considered to be inert and without effect on the actions of a drug dissolved in it. Glycofurol is a solvent suitable for the preparation of injectable solutions of many drugs, such as the benzodiazepines, vitamins, antibiotics, and other drugs with high oil-water partition coefficients. We are checking the effects of the solvent glycofurol on drug action. Our findings should give clues about the nature of the modifications of drug action which may be expected from the use of glycofurol-water solutions of drugs.

Proposed Course of Project: Enzyme induction by glycofurol will be compared with induction by phenobarbital sodium, dissolved in water, and by

3-methylcholanthrene, dissolved in corn oil. A study will be made of the modification of enzyme induction by the substitution of glycofurol-water solutions as the solvent for phenobarbital and 3-methylcholanthrene. The project will then be terminated after preparation of a manuscript for publication.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00895-02 LCP

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Studies of the Pathogenesis of Nitrofurantion-induced Lung Disease

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

PI:	H.A. Sasame	Chemist	LCP	NHLBI
OTHERS:	M. Boyd	Research Associate	OD	NHLBI
	J.R. Mitchell	Chief, Section on Clinical Pharmacology and Metabolism	OD	NHLBI

COOPERATING UNITS (if any)

Dr. M. Boyd is a Research Associate in the Pharmacology-Toxicology Program, NIGMS.

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme-Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	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)

In the 1976 annual report we described the development of an animal model for acute lung injury induced by nitrofurantion (NF). In the present report we describe studies of NF toxicity in rats which suggest that the drug may induce a form of pulmonary oxidant damage.

Project Description:

Objectives: The 5-nitrofuran derivative, nitrofurantion (NF), is widely used in the treatment of urinary tract infections. It is also used, on a more limited scale, for long-term prophylaxis against certain urinary tract pathogens. Unfortunately, the drug sometimes produces serious, and potentially fatal, lung disease in pstients. Both acute and chronic pulmonary reactions have been described. The acute form is characterized by a respiratory distress syndrome; chest X-rays usually show pulmonary infiltrates and effusions. The chronic reaction is more insidious in onset and clinical and radiologic manifestations are consistent with a diagnosis of pulmonary fibrosis. Lung biopsies have confirmed the fibrotic pulmonary changes.

Although these abnormal responses to NF have been widely assumed to represent hypersensitivity phenomena, immune mechanism have not been clearly implicated. Furthermore, neither the clinical spectra of the diseases, nor the organ specificities, are accountable simply as allergic reactions.

We have undertaken the present investigations to evaluate the possibility that NF produces a direct, dose-related pulmonary toxicity. We are also investigation the possibility that the toxicity involves the metabolism of the parent drug to a chemically reactive metabolite. We have chosen this approach since other nitrofurans are known to be converted to cytotoxic and carcinogenic species by nitroreduction; furthermore, many furans are metabolized to toxic metabolites that may produce lesions in livers, kidney, and lungs of experimental animals.

Methods Employed: Standard methods were used.

Major Findings: Rats raised from weanlings on a vitamin E-free diet containing high levels of polyunsaturated fat were markedly more susceptible to the pulmonary toxicity of NF (LD50 35 ± 10 mg/kg vs 400 ± 100 mg/kg). A three week period of dietary repletion with vitamin E, or change of the fat component to a saturated form, both reduced susceptibility fo the lesions. Exposure of either normal or vitamin E defizient rats to an atmosphere of 100% oxygen strongly potentiated pulmonary toxicity. In vitro studies revealed that lung contains enzymes that can reduce NF to chemically reactive intermediates which may either alkylate tissue components or react with oxygen to produce superoxide. In vivo studies suggest an inverse relationship exists between the generation of superoxide (and toxicity) and the alkylation of lung tissue by a metabolite of NF. All these studies suggest that metabolism of NF is required for its pulmonary toxicity and that the NF-induced lung lesions represent a form of oxidant lung damage.

Significance to Biomedical Research and the Program of the Institute: The observation of a reproducible and dose-related pulmonary toxicity with NF in experimental animals suggests that NF-induced lung disease in man may be the results of a direct pulmonary toxicity; it may, therefore, be dependent upon a variety of pharmacokinetic and constitutional factors. The model of NF-induced disease we described last year has been useful in elucidating factors, relation to the mechanism of the reaction and may also provide the basis for development of a more rational approach to the prevention and/or treatment of the disease in humans.

Proposed Course of Project: Further studies are underway to determine the mechanism of sensitization to NF toxicity produced by vitamin E deficiency in vivo. We are evaluating the possibility that vitamin E acts as an antioxidant to prevent NF toxicity or whether it affects the disposition and/or metabolism of NF. Detailed studies of the distribution and metabolism of NF in normal and vitamin E deficient rats are currently in progress.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00917-02 LCP

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Studies on the Formation of Reactive Metabolites of Phenacetin and Acetaminophen

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

PI:	J.A. Hinson	Staff Fellow	LCP	NHLBI
OTHERS:	S.D. Nelson	Staff Fellow	OD	NHLBI
	J.R. Gillette	Chief, Lab. of Chem. Pharm.	LCP	NHLBI

COOPERATING UNITS (if any)
Dr. S.D. Nelson is a Staff Fellow in the Section on Clinical Pharmacology and Metabolism.

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme-Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	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)

Phenacetin (PEAA) is converted by two different mechanisms to a reactive metabolite that is similar, if not identical to the reactive metabolite of acetaminophen (PHAA). 1) Incubation of hamster liver microsomes with PEAA, NADPH, and glutathione (GSH) in the presence of a $^{18}O_2$ atmosphere led to a 50% incorporation of ^{18}O into the para position of the PHAA-GSH conjugate, but no ^{18}O was incorporated into the conjugate when p-^{18}O-PHAA was incubated with the enzyme system. 2) When p-^{18}O-PEAA was incubated with the system in air, there was a 50% loss of ^{18}O in the PHAA-GSH conjugate, but no ^{18}O was lost when acetaminophen was incubated in the system. 3) When the N-O-glucuronide of PEAA was incubated in pH 7.4 buffer in $H_2{}^{18}O$, the ^{18}O was incorporated into the para position of the GSH conjugate. Thus there are three pathways for the formation of the reactive metabolite from PEAA. However, the mercapturates excreted in the urine after administration of ^{18}O-PHAA-PEAA showed no loss of ^{18}O in the metabolite derived from PHAA and only a 15% loss of ^{18}O in the metabolite derived from PEAA. Thus at least 70% of the mercapturate formed after the administration of PEAA was formed by way of PHAA.

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Project Description:

Objectives: Previous studies in our laboratory have shown that the toxicities of phenacetin and acetaminophen are mediated by reactive metabolites formed in the body, but the identity of the reactive metabolites are unknown. The objective of this study is to elucidate possible mechanisms for the formation of reactive metabolites formed from phenacetin and acetaminophen.

Methods Employed: Microsomes were isolated from hamsters by standard procedures. Using ^{18}O phenol as a starting material, methods were devised for the synthesis of p-^{18}O-acetaminophen with a 56±2% overall yield. From this p-^{18}O-acetaminophen, p-^{18}O-phenacetin was synthesized. The alternate nucleophile glutathione was used to trap the reactive species in large side incubations. Mass spectrometry was used to determine the structure of the ^{18}O content of the glutathione conjugates and the mercapturates. All other methods were standard procedures.

Major Findings: Phenacetin has been found to be converted by a liver microsomal cytochrome P-450-mixed function oxidase in hamsters to a reactive metabolite which covalently binds to protein. As has been previously shown with acetaminophen, glutathione blocks the covalent binding of acetaminophen and phenacetin and a glutathione conjugate is formed. When the glutathione conjugates derived from acetaminophen and phenacetin were analyzed by mass spectrometry both were glutathione-acetaminophen conjugates. Thus phenacetin had become deethylated. The mechanisms for formation of the reactive metabolites from phenacetin and acetaminophen, however, are different. The rate of formation of the reactive metabolite of acetaminophen was stimulated by pretreatment of animals with 3-methylcholanthrene, whereas the rate of formation of the reactive metabolite of phenacetin was stimulated by pretreatment of animals with phenobarbital. Glutathione trapping experiments in the presence of an $^{18}O_2$-atmosphere followed by analysis by mass spectrometry showed a 50% incorporation of ^{18}O into the reactive metabolite of phenacetin but no incorporation into the reactive metabolite of acetaminophen. When the reactive metabolite of p-^{18}O labeled phenacetin was trapped by glutathione and analyzed by mass spectrometry there was a 50% loss of ^{18}O. No loss of ^{18}O was observed when p-^{18}O-labeled acetaminophen was incubated and the glutathione conjugation isolated and analyzed. These data are consistent with the view that the reactive metabolite of phenacetin is formed by epoxidation and the 4,4-dihydroxyacetaminophen is an intermediate, whereas the reactive metabolite of acetaminophen is formed by N-hydroxylation followed by a spontaneous dehydration. Since the conversion of p-^{18}O-phenacetin to a reactive metabolite leads to loss of the ^{18}O label, the in vivo metabolism of p-^{18}O-phenacetin was studied. After injection of p-^{18}O-phenacetin, however, there was a 15% loss of the ^{18}O label in the mercapturic acid. These data indicate that conversion of phenacetin to a reactive metabolite may account for no more than 30% of reactive metabolites formed in hamster. Instead it seems likely that most of the mercapturic acid excreted after the administration of phenacetin is formed by way of acetaminophen.

Significance to Biomedical Research and Program of the Institute: The finding that the formation of reactive metabolites from acetaminophen and phenacetin occur by different mechanisms illustrates some of the complications that may arise in studies of drug toxicity.

Proposed Course of Project: The importance of these metabolites in the toxicities of phenacetin is being investigated.

Publication:

Hinson, J.A., Nelson, S.D. and Mitchell, J.R.: Studies on the microsomal formation of arylating metabolites of acetaminophen and phenacetin. Molecular Pharmacology, in press.

ANNUAL REPORT OF THE
LABORATORY OF CHEMISTRY
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

July 1, 1976, through September 30, 1977

As mentioned last year, the research of the Laboratory involves a variety of projects arising both within and outside of the Heart, Lung, and Blood Institute. The Laboratory now consists of three sections, the Section on Chemical Structure, the Section on Nuclear Magnetic Resonance and the Section on Physiological Chemistry.

The first two are almost purely chemical in nature and the identification of nmr as a separate section reflects the Laboratory's stress on this important area of structural investigation. We now possess two Fourier-transform multinuclear nmr spectrometers and both are in constant use by a wide group of investigators. We are progressing from gross structural analyses to investigations of more subtle details of mobile equilibria, conformation, and solvation.

In the area of mass spectrometry, we have consolidated our spectrometers in one room and acquired, on loan from LKB, a new LKB 2091 spectrometer with computer. This new spectrometer will allow us to quantitatively analyze a variety of biological intermediates by selected ion monitoring, a capability we have not had in the past and one which is much sought by NHLBI investigators.

In the area of X-ray crystallography, the use of newer computing methods developed by Dr. Silverton has resulted in even more rapid completion of structures and extension to compounds of even higher molecular weight. Last year he was able to complete 6 structures.

The NHLBI-EPA Chemical Information System now consists of 17 different spectral and bibliographic data bases including mass spectra, carbon-13 nmr, nmr and mass spectra bibliography, x-ray diffraction for organic and inorganic single crystals and powders, statistical analysis, and a substructure search system. Most of this system has now been made available to the public over a commercial computer system on a fee-for-service basis. It is used by over 300 scientists in North America and Europe.

Novel compounds whose structures have been elucidated by our Laboratory this year include benzoyl cyanide and an isomer of dolichodial from millipedes, isoamyl alcohol and acetate from bee stings, a series of phenylpentylisatins and -quinoline from a tumorogenic plants, an ethanolamine phosphoryl galactosylglycerol from Drosophila, a series of dialkylpyrrolidines from fire ant venom, atranorin from a rubber tree bark, justicidin B and polyacetylenic tetrahydropyrans and polythienyls from fish poisons, allergenic terpenes from Florida Holly, two new tetrahydroberberines from Ghanian plants, a variety of alkanols, ketones, monoterpenes, esters and aldehydes from formicine ants, two new hydroxyisocoumarins from beetles, cobalt tetraphenyl-porphyrin, gardineramulcine, imerubrine, a phenylselenide of codeine, a

dimethylketoglutarate condensation compound, an oxygen bridged dimer, "Beckman's Imide", 3,5-dipyrrolidinopyrrole, an isomer of pumiliotoxin C from frogs and last but perhaps most important biologically, a new metabolite of DOC found in urine of hypertensive rats, 19-nor-DOC.

ANNUAL REPORT OF THE
LABORATORY OF CHEMISTRY
SECTION ON PHYSIOLOGICAL CHEMISTRY
NATIONAL HEART, LUNG AND BLOOD INSTITUTE

July 1, 1976 through September 30, 1977

Kallikrein-Kinin System: High molecular weight (HMW) kininogen (also called Flaujeac, Williams, Fitzgerald or contact activation factor) in conjunction with plasma prokallikrein is required for the normal rate of activation of surface-bound Hageman factor. Turnover studies in rhesus monkeys with ^{125}I-labelled human and monkey low molecular weight and HMW kininogens purified by immunoaffinity and DEAE-cellulose chromatography showed similar half-life values by significantly different fractional catabolic rates. Human plasma kallikrein has been highly purified in five steps. Isoelectric focusing revealed four major peaks and pI's of 8.5, 8.8, 9.1, and 9.3, besides three minor variants. Human renal prokallikrein has been partially purified and characterized. Its molecular weight is 50,000, and that of the active enzyme, 35,000. Trypsin-activated prokallikrein is indistinguishable from renal kallikrein with respect to kininogenase activity. Assays were developed for urinary kinins and kininogen and applied to the assay of human urine samples and dog urine obtained in stop-flow studies. Urinary kallikrein probably originates in the distal tubule. Kinins are formed in the tubule and have a half-life in urine of about 3 hours. Cultured human endothelial cells (HEC) and smooth muscle cells were incubated with angiotensin I, bradykinin, angiotensin II, tetradecapeptide renin substrate and hog plasma renin subatrate to observe angiotensin converting enzyme, kininase, angiotensinase and renin-like activities. Converting enzyme (kininase II) was present only in HEC, but the other enzymes were found in both cell types.

Prostaglandins: The glutathione conjugate of prostaglandin A_1 (GSH-PGA_1) is much more actively reduced (up to 2.4 x 10^4 times) by PGE_2 9-ketoreductase than is PGE_2 the presumed natural substrate. The identity of GSH-9OH-PGA_1 was established by mass spectrometry. The reductase preferred GSH-PGA_1 over L-cysteine-PGA_1 and mercaptoethanolamine-PGA_1. These results indicate that GSH-PGA_1 or A_2 occurs naturally and is derived from PGA_1 or A_2 prostaglandins whose occurrence in man has been doubted. Prostaglandins B_1 and B_2, E_1 and E_2 have been separated by high performance liquid chromatography.

Determinants of Hormone Action: Vasopressin, Ca^{++}, carbechol, theophylline and ouabain failed to alter the rate of phospholipid turnover in rat renal cortical slices. Ranatensin markedly inhibited sodium transport in the toad bladder. The inhibition was not affected by atropine, but was blocked by pretreatment of submaximal doses of ouabain.

Villikinin: Villikinin has been further purified on an SP-Sephadex column. Two activity peaks were observed but attempts at further purification of each peak by cellulose thin-layer electrophoresis were unsuccessful due to poor recoveries from the plates.

SRS-A: A number of compounds were examined for their ability to increase the biosynthesis of SRS-A and for their possible incorporation into SRS-A. The

relationship of SRS-A to other mediators of bronchial asthma also was inves
gated.

Chemistry and Biology of Peptides: The gas chromatographic analysis of sil
lated dipeptides has been refined to the point where it is now possible to
analyze all dipeptides except those containing arginine. The purification
dipeptidylaminopeptidases I, IV and V has been refined to the point where
functionally pure I and IV can be prepared routinely. High-performance liq
chromatography, previously applied with impressive results for PTH-amino ac
analysis, has been extended to the analysis of dansyl and DNP amino acid
derivatives. A method has been developed for the micro detection of compou
with primary amino groups on thin-layer chromatography plates by dipping in
a solution of fluorescamine. A method has been developed for the micro ass
of histidine, histamine and related imidazoles based on the unique fluoresc
which develops after heating their fluorescamine derivatives in acid.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01002-05 LC

PERIOD COVERED

July 1, 1976, to September 30, 1977

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)

none

LAB/BRANCH

Laboratory of Chemistry

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

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)

The technique of Nuclear Magnetic Resonance spectroscopy is being applied to the study of the antibiotic Lasalocid A (X537A) and its binding to Serotonin and several cations to examine its function in membrane transport. The structure of compound 48/80 is being investigated by incorporation of enriched Carbon-13 paraformaldehyde.

PHS-6040
(Rev. 10-76)

Carbon-13 NMR binding studies of the ionophonic antibiotic Lasalocid A (x537A) with serotonin was initiated during the past year. Lasalacid A is known to increase membrane transport of cations and several other materials like serotonin. Compound X537A is known to exist as a monomer and several different types of dimers depending on the ions present and whether it is present in a hydrophobic or hydrophilic environment. Early experiments have centered on confirming assignments of various carbon resonances and defining whether dimers are present in the solutions. Spectra of the calcium, barium, and sodium salts show distinct differences in their proton resonances. Previous reports suggest this is due to the different dimers in equilibrium with the monomer. Carbon-13 spectra of Compound X537A with serotonin added show several resonances to be shifted from their original position, thus defining a sight for the binding interaction. Experiments are currently in progress to delineate the mode of binding interaction. This study is to be carried out in collaboration with Dr. John Costa (M-LES) and Dr. Colin Chignell (H-IR-PB).

A collaborative study with Dr. C. Chignell is underway on the Compound 48/80. This material formed from the polymerization of p-methoxy-N-methylphenethylamine and paraformaldehyde causes liberation of histamines from mast cells. Initial experiments were to confirm the proposed structure for this material, especially the sight of polymerization by incorporating ^{13}C enriched paraformaldehyde in the reaction. Literature on this material contains several possibilities, including cyclization of the phenethyl moiety to a tetrahydro isoquinoline before polymerization or incorporation of a chlorine into the polymer. The second possibility is ruled out by our data since no carbon is visible at a position consistent with this type substitution. The possibility of cyclization is still under investigation. Interestingly close examination of the carbon spectra shows that the methoxy carbon and the aromatic carbon to which it is bonded appear to have two resonance positions, which are tentatively explained as the carbons locked in the backbone of the polymer and the more mobile terminus.

Publications

1. Highet, R. J., Perold, G. W., and Sokoloski, E. A. Characterization of spiro-bislactonic phenolic metabolites of proteaceae by Carbon-13 nuclear magnetic resonance. J. Org. Chem., 41: 3860, 1976.

2. Shukla, Y. N., Sokoloski, E. A., Fales, H. M., and Kapadia, G. J. 6-Methoxy-7, 8-Methylenedioxycoumarin from Melochia tomentosa. Phytochemistry 15: 1788, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01003-06 LC

PERIOD COVERED

July 1, 1976, to September 30, 1977

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

COOPERATING UNITS (if any)

none

LAB/BRANCH

Laboratory of Chemistry

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	

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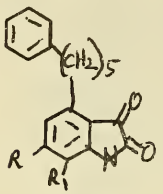
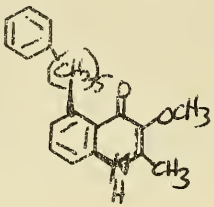
☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The structures of a variety of products found in biologically active plants, insect secretions, and other sources have been elucidated using mass spectrometry, nmr, and synthesis.

With G. Kapadia (Howard Univ.), the structures of several new alkaloids from the tumorogenic plant Melochia tomentosa have been elucidated. They contain the novel phenylpentyl isatin and -quinoline ring systems. Compound I





1.=R_1=OCH_3

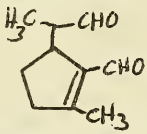
2. R=R_1=H

3. R=H, R_1= OCH_3

has been synthesized.

With M. Blum (Univ. of Georgia) the major volatile constituents expressed during bee stings have been identified as isoamyl alcohol and its acetate and benzyl acetate, along with lesser quantities of homologs. These are being tested for their effect in causing "swarming attacks" of the type exhibited by the so-called "killer bee" of Brazil.

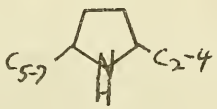
The structure of a new terpinoid defensive secretion found in Gastrophysa cyanea has been elucidated as a close relative of dolichodial.

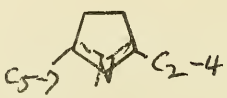


It is an extraordinarily powerful repellent of ants and perhaps other insects.

Benzoyl cyanide and mandelonitrile benzoate have been identified in the defensive secretion of millipedes. The former is a very reactive compound and has not been found in nature previously. It is also a potent ant repellent.

A series of cis and trans dialkylpyrrolidines and -pyrrolines have been characterized as the major components of the venom of the diminutive





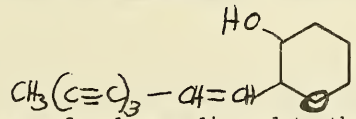
South African fire ant. The compounds have been synthesized. The corresponding piperidines from the much more toxic South American fire ants are occasionally allergenic, but the pyrrolidines appear not to be.

With L. Levenbook (NIAMDD), the structure of a compound responsible for rejection of second matings by fertilized drosophila has been elucidated as ethanolomine-O-4-galactosyl-1-glycerol.

With G. Wright (Univ. of Canterbury, N.Z.), evidence has been found for the retention of optical activity by gas phase ions. This new method using "meso ions" would allow demonstration of optical activity with the mass spectrometer in cases where one optical isomer was labelled and may be used in certain biosynthetic studies.

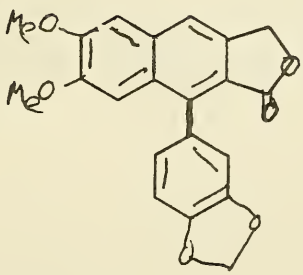
With H. Baer (FDA, Bureau of Biologics) the variation in urushiol structures within an individual poison ivy plant has been studied by examining individual leaves. Urushiols of different structures have widely different allergic effects in man and so varying responses may be obtained, depending on the point of contact even on one plant.

With D. Jerina (NIAMDD), T. Swain (Boston Univ.), T. Plowman and R. Schultes (Harvard), and N. Towers (U. of Brit.Col.) the principal investigator of this project spent March 11-May 15 in the Amazon region investigating local toxic plant and insect products. The structures of several poly-acetylene pyranes from Clibadium Sp. used as fish poisons were elucidated:

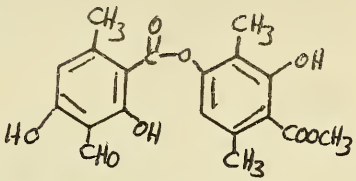


Other fish poisons found were di- and terthienyl

from Tessaria Sp. and justicidin B from Phyllanthus:



From the bark of a rubber tree, the useful antibiotic atranorin was identified:



An unusual compound of considerable biological potency in mice and humans has been isolated from Brunfelsia schultesi. Structural and biological investigations are underway.

Several novel, but as yet unidentified substances have been found in a series of formicine ants and stingless bees collected in the area.

Publications

1. MacConnell, J. G., Blum, M. S., Buren, W. F., Williams, R. N. and Fales, H. M. Fire ant venoms: Chemotaxonomic correlations with alkaloidal compositions. Toxicon. 14: 69-78, 1976.

2. Fales, H. M., Fenselau, C. and Duncan, J. H. The loss of water from isotopically labelled heptanals on chemical ionization. Org. Mass Spec. 11: 669-674, 1976.

3. Goren, M. B., Brokl, O., Roller, P., Fales, H. M., and Das, B. C. Sulfatides of Mycobacterium tuberculosis: The structure of the principal sulfatide. Biochemistry 15: 2728-2735, 1976.

4. Aldrich, J. R., Blum, M. S., Duffey, S. S., and Fales, H. M. Male specific natural products in the bug, Leptoglossus Phyllopus: Chemistry and possible function. J. Insect Physiol. 22: 1201-1206, 1976.

5. Shukla, Y. N., Sokoloski, E. A., Fales, H. M. and Kapadia, G. J. 6-Methoxy-7,8-Methylenedioxycoumarin from Melochia tomentosa. Phytochemistry 15: 1788, 1976.

6. Pedder, D. J., Fales, H. M., Jaouni, T., Blum, M. S., MacConnell, J., and Crewe, R. M. Constituents of the venom of a South African fire ant (Solenopsis punctaticeps) 2,5-dialkylpyrrolidines and -pyrrolines, identification and synthesis. Tetrahedron 32: 2275-2279, 1976.

7. Cagen, L. M., Fales, H. M., and Pisano, J. J. Formation of glutathione conjugates of prostaglandin A_1 in human red blood cells. J. Biol. Chem. 21: 6550-6554, 1976.

8. Duffey, S. S., Blum, M. S., Fales, H. M., Evans, S. L., Roncadori, R. W., Tiemann, D. L., and Nakagawa, Y. Benzoyl cyanide and mandelonitrile benzoate in the defensive secretions of millipedes. J. Chem Ecol., 3: 101-113, 1977.

9. Fales, H. M., and Wright, G. J.: Detection of chirality with the chemical ionization mass spectrometer-Meso" ions in the gas phase. J. Amer. Chem. Soc. 99: 2339, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01004-06 LC

PERIOD COVERED

July 1, 1976 to September 30, 1977

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)

none

LAB/BRANCH

Laboratory of Chemistry

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The purpose of this work is to find new natural compounds that may have physiological activity. New isocoumarins released with benzoquinones were discovered in the defensive secretions of the beetle Aspena pubescens. Potential carcinogens and chemical irritants in plant extracts of Sassafras, Acacia, Melochia and Schinus species were investigated. The structure of a new nor-steroid isolated from the urine of hypertensive rats was elucidated.

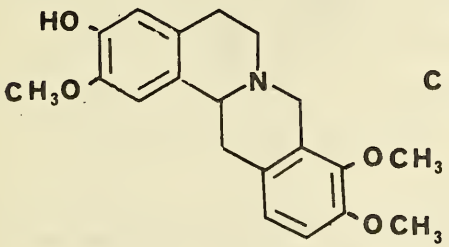
PHS-6040
(Rev. 10-76)

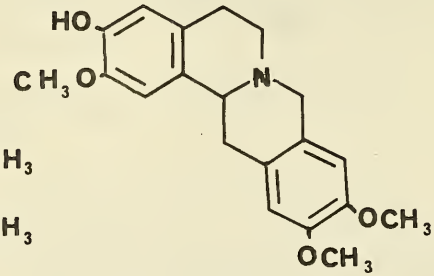
1. Plant Products

The search for components responsible for the allergies and respiratory ailments associated with the presence of Schinus terebinthifolius was continued. (in collaboration with Dr. Julia F. Morton, University of Miami). This South American tree introduced to Florida 50 years ago has spread rapidly, and is causing concern to the botanists, ecologists and health authorities of that state. Over fifty volatile terpenoids were isolated from the berries of Schinus and currently the flavones and biflavones of the plant are under study.

Plant substances of potential carcinogenic activity were studied (in collaboration with Dr. G. J. Kapadia, Howard University). The investigation of Melochia tomentosa, Acacia catechu, Sassafras albidum, Diospyros virginiaca was continued and the isolated compounds were examined by mass spectrometry.

A collaborative study (with Dr. Kwame Sarpong, University of Ghana) on the plant alkaloids of Ghana was initiated. The structures of several isoquinoline alkaloids were investigated by nuclear magnetic resonance and mass spectrometry. Two new tetrahydroberberine alkaloids were discovered:



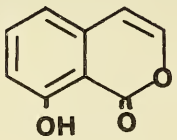


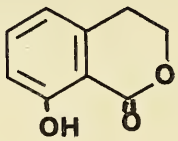
2. Insect Pheromones (in collaboration with Dr. M. S. Blum, University of Georgia).

The compositions of glandular secretions of ants and beetles were studied by combined gas chromatography-mass spectrometry, nmr, and high pressure liquid chromatography. Some of the new compounds found were synthesized for physiological testing.

Thirty five species of the ant genus Myrmecocystus were examined by GC-MS in an attempt to correlate the pheromone secretions with the taxonomic classification. A wide variety of compounds were detected. Among the main products were monoterpenes and n-alkanes, aldehydes (octanal, neral, geranial), 2-tri- and 2-pentadecanones, n-octanol, 2-tridecanol, 2,6-dimethyl-5-hepten-1-ol, 2,4-dimethyl-2-hexenoic acid, tridecyl esters of long chain acids, methyl anthranilate, etc. Some of the less commonly occurring compounds were synthesized for testing.

The defensive secretions of two beetles: Scaurus sp. and Apsena pubescens were investigated (in collaboration with Dr. W. R. Tschinkel, Florida State University). Besides methyl and ethyl benzoquinones which are widely used arthropod defensive substances, two new compounds, 8-hydroxy isocoumarin and 3,4-dihydro-8-hydroxyisocoumarin were found in the Apsena beetles.

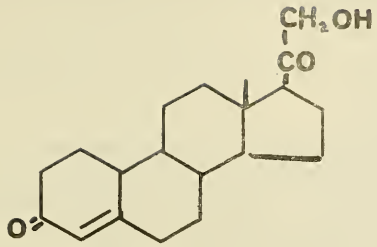




Their structures were inferred from a mass spectrometric study of the compounds and several derivatives. These isocoumarins have never before been found in nature nor have they been synthesized. In contrast with coumarins, the isocoumarins are not very common in nature. Their synthesis is in progress (with Dr. A. H. Khan, guest worker).

3. New Natural Nor-steroid (with Dr. Leon Milewich, Southwestern Medical School, University of Texas).

A few hundred micrograms of an unknown steroid were isolated from urine obtained from low renin hypertensive rats. It gave a blue tetrazolium test, had a Δ^4-3-oxo function and UV absorption maximum at 240 nm. The dimethoxime and dimethoximetrimethylsilyl derivatives of the steroid were prepared. Study of the mass spectra of the unknown steroid and its derivatives and the optical rotatory dispersion and circular dichroism curves, led to a 19-nor-deoxycorticosterone structure for the unknown.



This nor-steroid has never been isolated from biological or other natural sources. A synthesis of the compound is in progress.

Publications:

1. Bright, W. M., Lloyd, H. A., and Silverton, J. V. The crystal structure and absolute configuration of astrocasine methobromide. J. Org. Chem. 41: 2454-2458, 1976.

2. Chowdbury, B. K., Sethi, M. L., Lloyd, H. A., and Kapadia, G. J. Aporphine and tetrahydrobenzyl-isoquinoline alkaloids in Sassafras albidum. Phytochem. 15: 1803-1804, 1976.

3. Shukla, Y. N., Lloyd, H. A., Morton, J. F., and Kapadia, G. J. Iridoid glycosides and other constituents of Paederia foetida. Phytochem. 15: 1989-1990, 1976.

4. Kapadia, G. J., Shukla, Y. N., Morton, J. F., and Lloyd, H. A. New cyclopeptide alkaloids from Melochia tomentosa. Phytochem. 16: in press.

5. Lloyd, H. A., Jaouni, T. M., Evans, S. E., and Morton, J. F. Terpenes of Schinus terebinthifolius. Phytochem. 16: in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01005-06 LC

PERIOD COVERED

July 1, 1976 to September 30, 1977

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:	O. W. Adams	Guest Worker	ERDA
	H. Lingh	Student	Kennedy High School
	R. Highet	Research Chemist	CH NHLBI
	G. J. Shaw	Visiting Fellow	NHLBI

COOPERATING UNITS (if any)

none

LAB/BRANCH

Laboratory of Chemistry

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.3	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)

Crystallographic studies where the method provides unique answers or is the most rapid method of solving problems. Developement and use of Direct Methods for large ordered molecules

PHS-6040

Crystallographic studies where the method provides unique answers to problems of chemical and biological interest or is the most rapid method of solving such problems. Development and use of Direct Methods for large ordered molecules.

1. The crystal and molecular structure of triclinic cobalt tetraphenyl porphyrin.

This project, initiated last year with C. Kabuto, has been completed in collaboration with scientists of NIAMDD on spectroscopic and quantum mechanical aspects. The assessment of the combined results is in progress and has implications for studies of association of porphyrins in solution. Preliminary studies of other cobalt porphyrins have been carried out but no suitable crystals have been found as yet.

2. Gardineramulcine. This compound, molecular weight 812, is the largest molecule which has been studied by X-ray methods in this laboratory. The crystal structure had been attacked by earlier methods in other laboratories without success but has yielded fairly readily to methods developed here. Part of the problem may lie in the fact that it is extremely difficult to obtain a pure sample of this dimer of two similar but not identical molecules and the sample on which work has been carried out proves to be impure. The results are not vitiated but the not inconsiderable set of X-ray data (some five thousand measurements) has been remeasured on a new sample and refinement is in progress.

3. Extension of direct methods. The methods developed here are now being used, in collaboration with Dr. H. Ammon of the University of Maryland, to investigate a polysaccharide of molecular weight ca. 1400, which has resisted all other methods.

4. Imerubrine. A new aromatic alkaloid provided by Dr. M. Cava of the University of Pennsylvania. The crystal structure of this compound initially appeared simple but proved to be very difficult. The problem was finally solved by a unique application of direct methods. There are two molecules in the repeating unit and the crystallographic correspondence of the two molecules is very satisfactory. The molecule itself proves to be a unique fused tropolone ether.

5. The crystal structure and absolute configuration of a phenylselenide intermediate in the synthesis of (+) codeine. This problem represents an aspect of our work where the Xray method produces a unique answer in a shorter time than any comparable method. Despite the presence of the selenium atom, the results are as accurate as any morphinoid crystal structure which has ever been carried out.

6. The crystal structure of dimethylketoglutarate condensation compound (with G. John Shaw). This project represents a continuation of our collaborative work with Dr. U. Weiss of NIAMDD and has implications for possible simple steroid syntheses. The only remarkable point in this study, except for the structure iteself, was the total number of man-hours expended which is estimated to be 16. The project, of course, took longer than this but all other aspects were under computer control.

7. The structure of an oxygen-bridged cage dimer (with O. W. Adams). This compound, provided by Dr. Steven Bertz of Harvard University, proves to be a new type of cage compound.

8. The crystal and molecular structure of Peckman's Imide (with H. Lingh). While this compound has been known for 87 years and a chemical structure suggested, it is extremely intractable to chemical investigation and no structure proof existed prior to our work. The structure was solved readily with the assistance of Mr. Lingh (a high-school senior, entering MIT in the fall) and proves to have somewhat remarkable bond lengths. Quantum mechanical calculations on the structure were also carried out (again, with the assistance of Mr. Lingh) and further chemical and spectroscopic work is being pursued by Dr. R. Highet and Dr. Lin Tsai of NHLBI.

9. The crystal structure of 3,5-dipyrrolidinopyrrole (with R. Highet). The crystal structure has been solved but further work on interpretation of results is needed and is in progress.

10. The crystal structure of a red sulfur containing substituted indole (with O. W. Admas). While a chemical structure can be suggested for this compound, its absorption spectrum is unusual. The problem of solving the crystal structure has turned out to be somewhat difficult but progress has been made and further work is in progress.

Publications:

1. J. V. Silverton, P. M. Dansett, and D. M. Jerina. Oxidation of 4,5-dihydro-benzo[a]pyrene with osmium tetroxide. Stereochemistry of a substituted K-region diol. Tetrahedron Letters 1557, 1976.

2. W. M. Bright, H. A. Lloyd, and J. V. Silverton. Crystal structure and absolute configuraion of astrocasine methobromide. J. Org. Chem. 41: 2454, 1976.

3. M. Sato, H. Kon, H. Akoh, A. Tasaki, C. Kabuto, and J. V. Silverton. Anomalous magnetic properties of tetraphenylporphinato Co(II) complex in the solid state. Chemical Physics 16: 405, 1976.

4. J. V. Silverton, T. Akiyama, C. Kabuto, S. Sekita, K. Yoshihira, and S. Hatori. Xray analysis of Chaetoglobosin A, an indol-3-yl-[13]cytochalasan from Chaetomium globosum. Tetrahedron Letters: 1349, 1976.

5. C. Kabuto, J. V. Silverton, T. Akiyama, U. Sankawa, R. D. Hutchison, P. S. Steyn, and R. Vleggar. X-ray structure of viridicatumtoxin. A new class of mycotoxin from penicillium viridicatum westling. J. Chem. Soc. Chem. Comm: 728, 1976.

6. I. Iijima, K. C. Rice and J. V. Silverton. Studies in the (+)-morphinan series I. An alternate conversion of (+)-dihydrocodeinone into (+)-codeine. Heterocycles, in press 1977.

7. K. C. Rice, U. Weiss, J. V. Silverton, and G. J. Shaw. Reaction of 3-ketoglutarate with 1,2-dicarbonyl compounds V. Simple synthesis of derivatives of 2,3,2a,4,5,9b-hexahydro-1H-benz[e]indene from dimethyl 3-ketoglutarate and glyoxal. J. Org. Chem., in press 1977.

8. J. V. Silverton, C. Kabuto, K. T. Buck, and M. P. Cava. The structure of imerubrine, a novel condensed tropolone-isoquinoline alkaloid. J. Amer. Chem. Soc., in press 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NATIONAL INSTITUTES OF HEALTH INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01006-06 LC

PERIOD COVERED

July 1, 1976 - September 30, 1977

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
OTHER:	F.-T. E. Chou	Visiting Fellow	CH NHLBI

COOPERATING UNITS (if any)

none

LAB/BRANCH

Laboratory of Chemistry

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	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)

Dienone-Phenol Tautomerism: The tautomer of 3,5-dipyrrolidinophenol existing in aqueous solution has been shown to be the 2,4-dienone, with the position of equilibrium determined by hydrogen-bonding characteristics of the solvent.

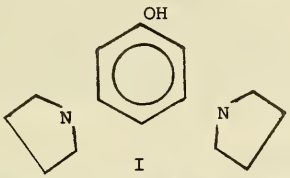
NMR: The C-13 nmr characteristics of Dendrobates alkaloids and allenic secoandrostantriones have been investigated.

Degradative Studies: A superior method for the characterization of unsaturated lactones by mass spectral means has been developed.

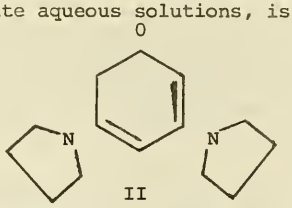
PHS-6040
(Rev. 10-76)

I. Studies of Tautomeric Systems

Studies of 3,5-dipyrrolidinophenol (I) in $CDCl_3$-CF_3CD_2OH solution show the structure characterized by absorption at 370 nm to be the 2,4-dienone II. This structure, which predominates in dilute aqueous solutions, is stabilized



I



II

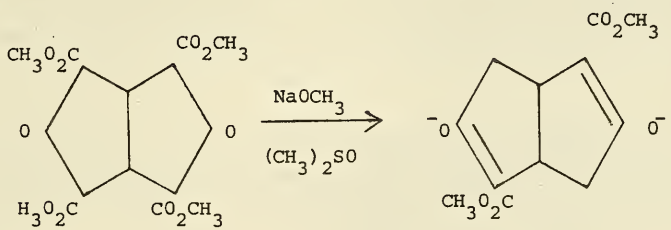
by the ability of the solvent to donate hydrogen bonds, while, in a competitive effect, the phenol I is favored by the solvent's ability to accept a hydrogen bond. The stability of II also reflects the unusual ability of the pyrrolidino moiety to donate electrons via Shiff base type structures, for 3,5-dimethylaminophenol exists in aqueous solutions as the corresponding dienone only to the extent of 30%, and no dienone can be detected of 3,5-dipiperidinophenol.

An x-ray crystallographic study of I, conducted in collaboration with J. V. Silverton of this Laboratory, has revealed that in the solid form only the phenolic form I can be detected.

II. The Characterization of Natural Materials by Nuclear Magnetic Resonance.

The availability of the 2-mm probe for the FX-60 nmr spectrometer has allowed the extension of structural studies to the skin alkaloids of the Colombian toad Dendrobates in collaboration with J. W. Daly of NIAMDD. The spectra recorded of eight alkaloids suggest that the minor alkaloid "219A" possesses the hydroquinoline system of pumiliotoxin C, but is isomeric at the sites of substitution.

A collaborative study with U. Weiss and S. Bhatnagar of NIAMDD has established that the reaction

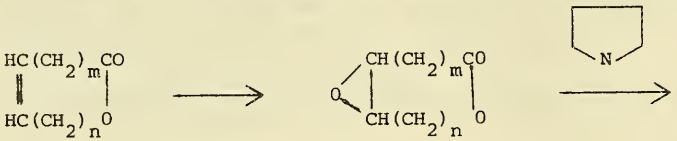


proceeds without detectable intermediate. The specificity is attributed to the charge separation afforded by the structure of the dianionic species.

A collaborative study with C. H. Robinson and D. A. Covey of Johns Hopkins University has assigned the carbon nmr resonances of the A-B seco allenic noradrostanones which inhibit Δ3-isomerase. In spite of the unusual conformation indicated by x-ray crystallographic examination, only the resonances of C-1,10 and 9 are affected by chilling to -80°C.

III. Degradative Studies on Unsaturated Lactones.

A superior degradative method has been developed to allow the characterization of submilligram quantities of unsaturated lactones by mass spectral techniques. Following the familiar epoxidation by m-chloroperbenzoic acid,



treatment by pyrrolidine provides a mixture of pyrrolidine carboxamides more cleanly than the previously used dimethylamine procedure. Fragmentation in the mass spectrometer occurs between the vicinal groups, with the chargebearing fragment that containing the amino groups. Such a study on the hyena lactone of molecular weight 224 shows m = 2, n = 9.

Publications:

1 Highet, R. J., Perold, G. W., and Sokoloski, E. A., Characterization of Spiro-bislactonic Phenolic Metabolites of Proteaceae by Carbon-13 Nuclear Magnetic Resonance," J. Org. Chem., 41: 3860-3862,1976.

2. Eppley, R. M., Mazzola, E. P., Highet, R. J., and Bailey, W. J., "Structure of Satratoxin H, a Metabolite of Stachybotrys alba. Application of ^{1}H and ^{13}C Nuclear Magnetic Resonance." J. Org. Chem. 42: 440-443, 1977.

3. Highet, R. J., and Chou, F-t. E., "The Dependence of Phenol-Dienone Tautomerism Upon the Hydrogen-Bonding Characteristics of the Solvent: 3,5-Dipyrrolidinophenol." J. Amer. Chem. Soc., 99: 3538-9, 1977.

4. Bhatnagar, S. P., Weiss, U., and Highet, R. J., "The Reaction of Dimethyl 3-Ketoglutarate with 1,2 Dicarbonyl Compounds VIII. Selective Base-Catalyzed Decarbomethoxylation of Tetramethyl cis-Bicylco[3.3.0] octane-3,7-dione-2,4,6,8-tetracarboxylate; Preparation of 2,6-Dicarbomethoxy-cis-bicyclo[3.3.0]octanedione." J. Org. Chem., 42: in press, 1977.

5. Highet, R. J., "The Use of Exchange with Deuterium Oxide as an Aid in the Characterization of Alcohols by Carbon13 Nuclear Magnetic Resonance," 172d Meeting of the American Chemical Society, Orgn 205 (1977).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01007-05 LC

PERIOD COVERED

July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Applications of Mass Spectrometry to Problems in Biochemistry

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

PI:	G. W. A. Milne	Research Chemist	LC NHLBI
OTHER:	G. J. Shaw	Visiting Scientist	LC NHLBI

COOPERATING UNITS (if any)

NIMH, Georgetown University, University of Bonn, W. Germany

LAB/BRANCH

Laboratory of Chemistry

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland, 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.3	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 uses of stable isotopes in biomedicine have been explored. Synthesis and behavior of deuterium and carbon-13 labelled alkanes, deuterium labelled serotonin and its congeners and deuterium labelled desipramine and imipramine have been recorded.

PHS-6040

Studies have continued of the use of stable isotopes and mass spectrometry in biomedical problems. Compounds labelled with stable isotopes are useful in the detection and quantitation of small amounts of the unlabelled material in biological samples. This method is particularly of value when the use of radioisotopes is contraindicated.

A large quantity of highly enriched 1,1,2,2-tetradeuteroserotonin has been synthesized. This material is not available commercially and consequently small samples of this material were made available to interested research workers. To date, eighteen 50 mg samples of this material have been distributed.

Further elaboration of this labelled serotonin was carried out to obtain the tetradeutero derivatives of N-acetylserotonin, melatonin, bufotenin and O-methylbufotenin. A few samples of these compounds have also been distributed.

In a parallel investigation, N-deuteromethylated desipramine and imipramine were synthesized for use in drug disposal and metabolism studies. Samples of these labelled materials were also made available to interested workers.

The role of L-valine as a precursor of the D-valine residue in actinomycin was studied with the help of 2,3-ditritio-L-valine that was specially synthesized for the purpose.

The mass spectral behavior of deuterium- and carbon-13 labelled n-dodecane has been studied in order to clarify the mechanism by which this molecule fragments under conditions of electron ionization.

Publications:

1. Shaw, G. J., Wright, G. J., and Milne, G. W. A.: The Synthesis of $\alpha,\alpha,\beta,\beta$-$d_4$-Serotonin. Biomed. Mass Spectrom., 3, 146, (1976).

2. Shaw, G. J., Wright, G. J., and Milne, G. W. A.: Mass Spectra of Some Specifically Deuterated Tryptamines. Biomed. Mass Spectrom., 4, ppp, (1977).

3. Shaw, G. J., and Markey, S. P.: The Synthesis of N-deuteromethylated Desipramine and Imipramine. Biomed. Mass Spectrom., 4, ppp, (1977).

4. Mason, K. T., Shaw, G. J., and Katz, E.: Biosynthetic Studies with L-2,3-$^3H_2$) Valine as Precursor of the D-valine Moiety in Actinomycin. Biochim. Biophys. Acta, ppp, (1977).

5. Shaw, G. J., and Milne, G. W. A.: Synthesis and Properties of Dodecane-,12-$^{13}C_2$ and Dodecane-1,1,1,12,12,12-$^2H_6$. J. Label. Compounds 12, 557 (1976).

6. Levson, K., Heimbach, H., Shaw, G. J., and Milne, G. W. A.: Isomerization of Hydrocarbon Ions. VIII. The Electron-impact Induced Decomposition of n-Dodecane. Org. Mass Spectrom., 12, ppp, (1977).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01008-06 LC

PERIOD COVERED

July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

The Use of Digital Computing in Problems in Biochemistry

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

PI:	G. W. A. Milne	Research Chemist	LC NHLBI
Other:	E. Subramanian	Visiting Associate	LC NHLBI
	S. R. Heller	Computer Specialist	EPA-MIDSD

COOPERATING UNITS (if any)

EPA, NBS, ERDA, FDA, NSF, Agencies in U.K., France, Switzerland, Holland, Germany, Hungary, Japan

LAB/BRANCH

Laboratory of Chemistry

SECTION

INSTITUTE AND LOCATION

NHLBI-NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.2	2.2	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)

The NIH-EPA Chemical Information System, comprising 17 different components such as spectral data and bibliographic data bases, is under systematic development in a project that involves the collaborative efforts of 36 different groups in the U.S., Europe, and Japan.

Components of this system include a mass spectral search system, a carbon-13 nmr search system, nmr and mass spectral bibliographic search systems, a structure search program, searchable data bases of X-ray diffraction data for organic crystals, inorganic crystals and powders and a number of data base-independent programs.

The entire system is being delivered to members of the international scientific community via networked computers in the private sector. All who use the system do so on a fee-for-service basis.

3/7

PHS-6040
(Rev. 10-76)

The NIH-EPA Chemical Information System (CIS) is currently being supported by six agencies of the U.S. government and involves the active collaboration of 36 different groups in 10 different countries.

Components of the CIS that are now operating in the private sector include the mass spectral search system, the carbon-13 nmr search system, a mass spectral literature search system, a statistical analysis package, a search system for X-ray diffraction data for organic compounds, and a substructure search system. A total of more than 300 scientists in North America and Europe use one or more of these systems.

In the development phase at NIH, there are a further 11 components which are currently being brought up to a sufficiently reliable level for export to a public computer network. These newer components include an inorganic crystal data base, a file of powder diffraction data, a number of thermodynamic property files such as gas phase proton affinities and heat of formation data.

Publications:

1. Heller, S. R., Milne, G. W. A., and Feldmann, R. J.: A computer-based chemical information system. Science, 195, 253, (1977).

2. Milne, G. W. A.: Review of eight peak index of mass spectra, compilation of mass spectral data, and registry of mass spectral data. Biomed. Mass Spectrom., 4, 68 (1977).

3. Heller, R. S., Milne, G. W. A., Feldmann, R. J., and Heller, S. R.: An international mass spectral search system (MSSS). Part V. A status report. J. Chem. Inf. & Comp. Sci., 16, 176 (1976).

4. Milne, G. W. A., and Heller, S. R.: Quality control of chemical data bases. J. Chem. Inf. and Comp. Sci., 16, 321 (1976).

5. Milne, G. W. A., and Heller, S. R.: The MSDC-EPA-NIH mass spectral search system. Amer. Lab., 8, 43 (1976).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01010-03 LC

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Biosynthesis of the Slow Reacting Substance of Anaphylaxis (SRA-A) From Monkey Lung

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

PI:	M.E. Webster	Senior Investigator	
OTHER:	B.D. Davis	Chemist	LC NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Physiological Chemistry

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	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)

SRS-A (Slow Reacting Substance of Anaphylaxis) was first described in 1940 and shown to be released from monkey lung in 1966. Although SRS-A is well known as a mediator of bronchial asthma, the biochemical mechanism of its synthesis and the enzymes involved in its formation remain unknown. In this study a number of compounds were examined for their ability to increase the biosynthesis of SRS-A and for their possible incorporation into this biologically active compound. The relationship of SRS-A to other mediators of bronchial asthma was investigated.

PHS-6040
(Rev. 10-76)

Objectives: Slow Reacting Substance of Anaphylaxis (SRS-A) was first described in 1940 and shown to be released from monkey lung in 1966. Although SRS-A is well known as a mediator of bronchial asthma, the biochemical mechanism of its synthesis and the enzymes involved in its formation remain unknown. In this study a number of compounds have been examined for their ability to increase the biosynthesis of SRS-A and for their possible incorporation into this biologically active compound. The relationship of SRS-A to other mediators or bronchial asthma has been investigated.

Methods Employed: Monkey lung fragments (50-100 mg) were sensitized with human ragweed antisera, washed free of serum proteins and incubated for 1-60 minutes at 37°C with varying concentrations of antigen E (5-5000 ng). The biological activity of SRS-A was determined by bioassay utilizing the isolated guinea pig ileum in the presence of anticholinergic and antihistaminic agents. Histamine and arginine esterase were determined by radiochemical techniques described elsewhere (Webster et al., Ciencia e Cultura 26: 372, 1974; #Z01 HL 1945-03 HE, 1975).

Major Findings: Previously our laboratory described the release of SRS-A, histamine, and arginine esterase from monkey lung and human lung sensitized with human ragweed antibody and challenged with specific antigen. (Z01 HL 01011-03 LC, 1976; Z01 HL 01010-02 LC, 1976). Further attempts to develop a biochemical method for the determination of SRS-A have been unsuccessful. Although SRS-A probably inhibits arylsulfatase activity when p-nitrocatechol sulfate is used as substrate, the sulfate, phosphate, and chloride ions present in crude tissue supernatants also inhibit the enzyme. Detailed studies indicated that SRS-A does not contain thiol or disulfide groups. With thin layer chromatography on silicic acid or DEAE-cellulose plates, SRS-A activity was completely lost. Although SRS-A could be measured following extraction with organic solvents, the activity decreased rapidly. Therefore, SRS-A activity in our studies was measured in bioassays by contraction of the guinea pig ileum.

The mast cell is the source of mediators such as histamine, but studies of the various cellular elements involved in SRS-A synthesis have produced conflicting evidence, with polymorphonuclear leukocytes, basophils, or mast cells implicated by different investigators. We examined the relationship among SRS-A, histamine, and arginine esterase in 24 experiments with lungs from 2-3 year old rhesus monkeys. Earlier studies in our laboratory (Z01 HL 01010-02 LC, 1976) had shown that lungs from the rhesus monkey usually generate more mediators than those derived from cercopithecus aethiops (African green) and that lungs from younger animals release larger quantities of mediators. By multiple regression analysis, no significant relationship among SRS-A, histamine and arginine esterase could be detected. For SRS-A and histamine, the coefficient of correlation was only .436 for a linear curve ($P < .05$); similarly, coefficients for SRS-A and arginine esterase and for histamine and arginine esterase indicated little correlation. These data suggest that although mast cells may be involved in SRS-A generation, SRS-A is not generated by the same mechanism as histamine.

The relationship between sex of the animal and release of mediators was also studied. Generation of SRS-A and arginine esterase was similar in lungs from male and female monkeys, but lungs from males released twice as much histamine as lungs from females.

A large number of sulfur-containing compounds and carboxylic acids have now been tested for their effect on the generation of SRS-A and histamine in monkey lung supernatants. Sodium sulfide, methionine, and cysteine increased SRS-A production by 17-72%. The C- and L- forms of both cysteine and methionine were equally stimulatory, suggesting that these compounds do not increase SRS-A synthesis by direct incorporation into enzymes or substrates involved in SRS-A synthesis. An increase in histamine usually paralleled that of SRS-A, although the amounts of the increase sometimes differed quantitatively. In preliminary experiments with other sulfur-containing compounds, reduced and oxidized glutathione and taurine usually enhanced SRS-A and histamine generation. Sulfate, however, inhibited generation of the two compounds, while homocysteine in both the free acid and lactone forms inhibited SRS-A release and homocystine inhibited the release of histamine. Sodium bisulfite inhibited SRS-A release completely but greatly increased histamine release.

Of the carboxylic acids, succinic acid, α-ketoglutaric acid, and probably maleic acid were particularly effective in enhancement of SRS-A generation, with increases of 40-123%. SRS-A generation was stimulated slightly by citric acid, acetic acid, and glutaric acid. Histamine release usually increased also. On the other hand, malic acid caused a reduction in SRS-A generation but did not affect histamine release.

The combination of succinic acid and sulfide yields even greater amounts of SRS-A, indicating that the two compounds increase SRS-A activity by different mechanisms. Furthermore, different preincubation times were required for optimal SRS-A generation with sulfide and succinic acid. Preincubation for 5 minutes with sulfide or methionine produced the greatest formation of SRS-A. With succinic acid, however, greater formation of SRS-A was obtained with shorter preincubation periods.

Preliminary evidence indicates that SRS-A generated in the presence of sodium sulfide may have different chemical properties from that generated with succinic acid. SRS-A generated in the presence of sulfide, like fractions II, III, and IV of human SRS-A, could not be extracted into chloroform, whereas SRS-A generated either in the presence of succinic acid alone or in the absence of both enhancing compounds was extracted into chloroform.

The possibility that sulfide and succinic acid are precursors of SRS-A was studied with labeled compounds but experiments were inconclusive. SRS-A could be separated from over 90% of the labeled succinic acid by extraction with chloroform but a small quantity of radioactivity remained in the organic layer. Although the peak of sulfide radioactivity was separated from the SRS-A peak by LH-20 chromatography, all of the SRS-A fractions contained radioactive sulfide. Further investigation is necessary to determine the precursors of SRS-A and to establish whether SRS-A is more than one compound.

Significance to Biomedical Research and the Program of the Institute: Investigation into the mechanism of biosynthesis of monkey SRS-A should assist in the determination of the structure of SRS-A and in the development of antagonists to SRS-A which would be of therapeutic value in bronchial asthma.

Proposed Course of Project: The project will be discontinued.

PROJECT NUMBER (See instructions for Z01 HL 01012-04 LC)

HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

Z01 HL 01012-04 LC

PERIOD COVERED
July 1, 1976 to June 30, 1977

TITLE OF PROJECT (80 characters or less)
Amino Acid Sequence Determination of Polypeptides

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

PI:	J.J. Pisano	Head, Sect. on Physiological Chem. LC NHLBI
OTHER:	H.C. Krutzsch	Staff Fellow

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemistry

SECTION
Section on Physiological Chemistry

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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)

Research has continued on the dipeptidyl aminopeptidase (DAP) method for polypeptide sequencing using gas chromatography-mass spectrometry (GC-MS) for dipeptide identification. The majority of work was focused on the enzymology of the method. Purification techniques were developed for the purification of DAP I, IV and V. Hopefully, all polypeptides now will be degraded. The use of the DAP/GC-MS method appears to be a superior means for sequence analysis. Sensitivity and speed are the major assets of the technique.

Objectives: To increase the sensitivity and speed of polypeptide sequencing through the use of dipeptidyl aminopeptidases (DAP) to degrade polypeptides and gas chromatography-mass spectrometry (GC-MS) to determine the released dipeptides.

Methods Employed:

(1) DAP digestion of the polypeptide and its single Edman-degraded congener.

(2) GC-MS of the volatile dipeptide derivative mixtures for separation and identification of the dipeptides.

(3) Combination of the two sets of dipeptide fragments to yield the structure of the polypeptide.

Major Findings: A major effort was focused on the enzymology of the DAP/GC-MS polypeptide sequencing method. As the polypeptide digestion is now carried out, up to three DAP's are used for complete digestion, DAP I, IV and V. DAP I cleaves all peptide bonds except those involving proline. DAP IV cleaves the carboxy side of Pro and DAP V cleaves on the imino side of Pro.

The process for preparation of DAP I was redefined and simplified to reproducibly allow the high yield production of functionally pure enzyme with high specific activity. Using methods developed in other laboratories, preparation of DAP I yielded material with contaminating proteases.

After extraction from beef spleen and ammonium sulfate precipitation, DAP I is heat-treated and further purified by gel filtration. The active fractions are again heated for final purification. The mercaptan requirement for DAP I is now fulfilled by 2-methoxyethyl mercaptan instead of mercaptoethanol. The former gives very low background in the gas chromatograph.

The method previously reported for obtaining DAP IV also was modified to give functionally pure, high specific activity enzyme in good yield. DAP-IV is extracted from hog kidney, then precipitated with ammonium sulfate. The procedure now has three column steps: 1) DEAE-cellulose, 2) gel filtration and 3) affinity chromatography to remove dipeptidase activity. The enzyme from this preparation was tested with a number of X-Pro-B-naphthylamide substrates, where X was all amino acid residues except Cys. The results indicated that DAP IV possessed a broad activity toward cleavage of amide bonds involving the carboxyl group of proline. Unlike DAP I and IV, DAP V has no unique characteristics, such as heat or pH stability, salt or solvent precipitation, or column migration, that would facilitate its purification. Thus, the purification of DAP V presently requires four column steps after extraction from hog kidneys and ammonium sulfate precipitation. These are: 1) CM-Sepharose-CL-6B, 2) a DEAE-type Sepharose-CL-6B prepared from carbodiimide coupling of ethylene diamine to CM-Sepharose-CL-6B, 3) hydrophobic affinity chromatography with a commercially available packing, and 4) gel filtration. The special column packing for step 2 was made because DAP V irreversibly binds to the usual, more hydrophobic DEAE-Sepharose-CL-6B.

Attempts to take advantage of the hydrophobic affinity of this enzyme failed with columns prepared with cyanogen bromide and an aliphatic amine because of irreversible binding to the ureido (-O-C(=NH)-NHR) linkage, but were successful when an ether link was used to bind the hydrophobic group to the resin. In the course of purifying DAP I, IV and V, eight new proteolytic activities have been tentatively identified. These include two DAP V's, three monoaminopeptidases, two endopeptidases and an aminotripeptidylpeptidase. DAP V also has been partially characterized. It has a molecular weight of 160,000 to 200,000, a pH optima in the range of 7 to 8, and is stable to approximately 50 degrees at pH 7. It is not inhibited by mercaptans, PMSF, iodoacetamide, or phenylmercuric chloride, requires manganese ion and is inhibited by EDTA and o-phenanthroline.

Further work also was done in the area of gas chromatography of trimethylsilylated dipeptides. A column packing (1% OV-1 on 80-100 mesh Chromosorb 750) has been found that allows detection of all dipeptides except those containing Arg. Even dipeptides such as His-Gln, Asn-Trp and Gln-Trp are eluted. This packing has been tested with approximately 25 pure dipeptides, containing all types of side chain functional groups. The data indicated that approximately equal responses are obtained at the 5 nmolar level, and that approximately 0.1 nmole of dipeptide is the minimum amount that can be detected. Dipeptides containing Arg can be detected if the guanido group is removed with hydrazine. Successful GC analysis of these types of dipeptides was also possible following DAP digestion when the polypeptide was treated with hydrazine prior to digestion.

Significance to Biomedical Research and the Program of the Institute: The work described above will significantly increase the scope, utility and sensitivity of polypeptide sequencing. The method will help to provide the required sensitivity and confidence in the elucidation of the structures of small quantities of biologically important polypeptides, such as enzymes, transplantation antigens, hormones, cell growth factors, etc.

Proposed Course: Future work will center on completion of the purification of DAP V. Because DAP IV is also derived from the same source, efforts will be made to integrate as much as possible the simultaneous preparation of both these enzymes. The new sequencing methodology will be extensively applied to document its utility.

Publications:

Krutzsch, H.C. and Pisano, J.J.: Identification of Dipeptides by Gas Chromatography-Mass Spectrometry and Application to the Dipeptidyl Aminopeptidase Approach to Polypeptide Sequencing. Methods in Enzymology, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01014-07 LC

PERIOD COVERED

July 1, 1976 to June 30, 1977

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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	C.L. Zimmerman	Chemist	LC NHLBI
	E. Appella	Medical Officer (Biochemist)	LCB NHLBI

COOPERATING UNITS (if any)

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LAB/BRANCH

Laboratory of Chemistry

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Section on Physiological Chemistry

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NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.4	1.4	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

PTH amino acids are now routinely analyzed in less than 20 minutes on a 25 x 0.46 cm column of Zorbax ODS (DuPont).

A high performance liquid chromatographic (HPLC) method has been developed for the analysis of histidine containing dipeptides, methyl-histidines, polyamines and related amino acids.

HPLC has been applied to the analysis of Dansyl (N,N-dimethylnapthylenesulfonyl chloride) and DNP (fluorodinitrobenzene) derivatives of amino acids.

PHS-6040
(Rev. 10-76)

Objectives: To develop methodology for the micro characterization and determination of proteins, peptides and amino acids.

Methods Employed: High performance liquid chromatography (HPLC) spectrophotofluorometry, automated and manual Edman degradation of polypeptides.

Major Findings: All 20 amino acid phenylthiohydantoins (PTH's) can be separated in a single analysis in less than 20 min using a 25 x 0.46 cm DuPont Zorbax ODS column developed with a gradient of acetonitrile in 0.01 M sodium acetete buffer, pH 4.5. With the single solvent, 0.01 M sodium acetate (pH 4.5)-acetonitrile, 42:58 (v/v), all PTH's are separated in less than 10 min except serine and glutamine, which coelute, and arginine which elutes at about 14 min. With this procedure it is possible to keep pace with automated Edman methods.

Analysis of several histidyl dipeptides and polyamines found in brain has been achieved by HPLC using a strong cation exchange column (partisil 10-SCX, Whatman) and lithium citrate buffers. Quantitation (to 10 pmoles) was achieved using O-pthaladehyde reagent and fluorescent detection.

Several dansyl and DNP amino acids can be resolved in 25 minutes by HPLC using a column of Zorbax ODS (DuPont Instruments) and a gradient from 1% acetic acid to acetonitrile. UV detection allows quantitation in the subnanomole range.

Significance to Biomedical Research and the Program of the Institute: Laboratories involved in the isolation, purification and amino acid sequence analysis of polypeptides will benefit from the speed and sensitivity of the above methods.

Publications:

Zimmerman, C.L., Appella, E. and Pisano, J.J.: Advances in the Analysis of Amino Acid Phenylthiohydantoins by High Performance Liquid Chromatography. Anal. Biochem. 75:77-85, 1976.

Zimmerman, C.L., Appella, E. and Pisano, J.J.: Rapid Analysis of Amino Acid Phenylthiohydantoins by High-Performance Liquid Chromatography. Anal. Biochem. 77:569-573, 1977.

Zimmerman, C.L. and Pisano, J.J.: High Performance Liquid Chromatography of Amino Acid Derivatives. In C.H.W. Hirs (Ed.) Methods in Enzymology, Part E, Enzyme Structure., In press.

Nakamura, H. and Pisano, J.J.: Fluorescamine Derivatives of Histidine, Histamine, and Certain Related Imidazoles: Unique Fluorescence After Heating in Acid. Arch. Biochem. and Biophys. 177:334-335, 1976.

Nakamura, H. and Pisano, J.J.: Detection of Compounds With Primary Amino Groups on Thin-Layer Plates by Dipping in a Fluorescamine Solution. J. Chrom.

121:79-81, 1976.

Nakamura, H. and Pisano, J.J.: Derivatization of Compounds at the Origin of Thin-Layer Plates With Fluorescamine. J. Chrom. 121:33-40, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01015-06 LC

PERIOD COVERED
July 1, 1976 to June 30, 1977

TITLE OF PROJECT (80 characters or less)

Characterization of Villikinin

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

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	Eszter Kokas		Univ. of N.C. Medical School

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Villikinin is a 2000 m.w. polypeptide which has the unique action of causing a relatively prolonged pumping of dog intestinal villi when injected I.V. Further purification of porcine villikinin was attempted. A substance with properties similar to mucosal villikinin has been isolated from urine.

Objectives: To isolate and determine the structure of villikinin, a putative gastrointestinal hormone obtained from intestinal mucosa which has a specific stimulant action on intestinal villous motility.

Methods Employed: Canine and porcine intestinal mucose samples were extracted with trichloroacetic acid and the extracts applied to Dowex 50, Bio-Gel P-4 and P-10 columns. Further purification was achieved using droplet counter-current chromatography, ion exchange chromatography on SP-Sephadex, and electrophoresis on cellulose thin-layer plates. The activity of the fractions was determined by bioassay in vivo, by the usual method of noting the increase in intestinal villous motility.

Major Findings: Two peaks of activity were recovered when 700 units (see report Z01 HL 01015-05 LC) villikinin were chromatographed on an SP-Sephadex column using a gradient of pH 5.0 ammonium acetate buffer from 0.05 to 0.2 M. Each peak was separately electrophoresed on cellulose thin-layer plates at 2000 V for 30 min using a pyridine acetate buffer pH 3.5. The recovery of activity from the plates was poor for both peaks.

Significance to Biomedical Research and the Program of the Institute: Villikinin appears to be a specific hormone which causes intestinal villi to contract. This pumping of the villi promotes lymph flow and, presumably, absorption of nutrients from the GI tract.

Proposed Course: Attempts to isolate sufficient villikinin for sequence analysis will be abandoned. Further characterization of the peptide is planned.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01016-07 LC

PERIOD COVERED
July 1, 1976 to June 30, 1977.

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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COOPERATING UNITS (if any)
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LAB/BRANCH
Laboratory of Chemistry

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NHLBI, NIH, Bethesda, Maryland 20014

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1.5	1.5	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A radioimmunoassay for urinary kinins was developed and applied to the assay of kinins in human urine. Prekallikrein and urokininogen were discovered in human urine. Prekallikrein probably arises from the distal tubule. The half-life of kinins in urine is 2-4 hours.

Objectives: To develop procedures for the assay of components of the kallikrein-kinin system. To establish the role of the kallikrein-kinin system in health and disease.

Methods: Bioassay and radioimmunoassay of kininogen, kininases, and kinins. Enzymatic digestions of urine with trypsin and kallikrein. Radiochemical assay of kallikrein. Stop-Flow measurements of the dog kidney.

Major Findings:

Radioimmunoassay of Kinins in Urine

A rapid and sensitive radioimmunoassay for total urinary kinins (bradykinin, lysyl-bradykinin, and methionyl-lysyl-bradykinin) has been developed. As little as 1.2 ug/ml can be determined. Kinin fragments including residues 1-4, 1-6, 1-7, 4-7, 3-7, 2-7 did not cross-react. However, fragment 1-8 has 1/400 and kininogen 1/20 the activity of bradykinin, respectively. The kinins in 10 samples determined by RIA was 20.9 ± 2.7 ug/24 hrs (mean ± SEM) and by bioassay 19.7 ± 2.7. The correlation coefficient of the linear regression line was 0.86 ($P<0.001$).

Urokininogen

When seven urine samples were incubated with trypsin, substantial kinin was generated in every case. The kinin content increased 61 ± 7% (mean ± SEM).

Normally, kininogen is slowly hydrolyzed in urine. In a test of light samples, kininogen decreased 48 ± 14% (mean ± SEM) after three hours incubation of fresh urine. This slow hydrolysis rate may be due in part to the partial inhibition of kallikrein in urine as only 64 ± 5% of human urinary kallikrein added to urine is recovered.

Kininase

In six urine specimens 38 ± 6% (mean ± SEM) of the kinin disappeared in two hours and in four hours 41-82% disappeared. The highest rates of disappearance occurred in the urine of samples closer to neutrality.

Prekallikrein

After trypsin digestion of 10 urine samples, the level of kallikrein increased 57 ± 5% (mean ± SEM). The trypsin-activable kallikrein is probably prekallikrein as it could be separated from active kallikrein and has an apparent molecular weight of 50,000 as compared with 35,000 for active kallikrein.

Stop flow studies (in two dogs) reveal a peak of kallikrein activity indicative of the distal tubular organ of urinary kallikrein. On the other hand kinin and urokininogen were found in all stop-flow fractions. Studies are in progress to determine where kininogen enters the nephron and where kinins are formed.

Publications:

Geller, R.G., Yoshida, H., Beaven, M.A., Horakova, Z., Atkins, F.L., Yamabe, H.,

and Pisano, J.J.: Pharmacologically Active Substances in Venoms of the Bald-Faced Horner, *Vespula (Dolichovespula) Maculata*, and the Yellow Jacket, *Vespula (Vespula) Maculifrons*. Toxicon 14: 27-33, 1976.

Hial, V., Keiser, H.R., and Pisano, J.J.: Origin and Content of Methionyl-Lysyl-Bradykinin, Lysyl-Bradykinin and Bradykinin in Human Urine. Biochem. Pharmacol. 25: 2499-2503, 1976.

Imanari, T., Kaiqu, T., Hisanobu, Y., Kerin, Y., Pierce, J.V., and Pisano, J.J.: Radiochemical Assays for Human Urinary, Salivary, and Plasma Kallikreins. In Pisano, J.J. and Austen, K.R. (Eds.): Chemistry and Biology of the Kallikrein-Kinin System in Health and Disease. U.S. Government Printing Office, Washington, D.C., 1976, pp. 205-213.

Imanari, T. Wilcox, G.M., and Pisano, J.J.: Sensitive Radiochemical Esterolytic Assays for Urokinase. Clin. Chim. Acta 71: 267-276, 1976.

Keiser, H.R., Geller, R.G., Margolius, H.S., and Pisano, J.J.: Urinary Kallikrein in Hypertensive Animal Models. Fed. Proceed. 35: 199-202, 1976.

Yoshida, H., Geller, R.G., and Pisano, J.J.: Vespulakinins: New Carbohydrate-Containing Bradykinin Derivatives. Biochem. 15: 61-64, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01017-06 LC

PERIOD COVERED

October 1, 1976 through April 15, 1977

TITLE OF PROJECT (80 characters or less)

Metabolism of Prostaglandin A

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

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OTHER:	L.M. Cagen	Staff Fellow	LC NHLBI
	H.M. Fales	Chief, Lab. of Chemistry	LC NHLBI
	R.E. Bowden	Clinical Associate	HEB NHLBI
	M.P. Peyton	Chemist	LC NHLBI
	C.L. Zimmerman	Chemist	LC NHLBI

COOPERATING UNITS (if any)

Hypertension-Endocrine Branch, NHLBI, NIH, Bethesda, Maryland

LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Physiological Chemistry

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

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)

The glutathione conjugate of prostaglandin A_1 (GSH-PGA_1) is much more actively reduced (up to 2.4×10^4 times) by PGE_2 9-ketoreductase than is PGE_2 the presumed natural substrate. The identity of GSH-9OH-PGA_1 was established by mass spectrometry. The reductase preferred GSH-PGA_1 over L-cysteine-PGA_1 and mercaptoethanolamine-PGA_1. These results indicate that GSH-PGA_1 or A_2 occurs naturally and is derived from PGA_1 or A_2, prostaglandins whose occurrence in man has been doubted.

Prostaglandins B_1 and B_2, E_1 and E_2 have been resolved by high performance liquid chromatography using a 0.46 x 25 cm Zorbax ODS column and isocratic elution with methanol:0.1% formic acid, 70:30.

PHS-6040

Objectives:

(1) To test the feasibility of the selective reaction of GSH with PGA for the assay of urinary PGE_2 by high performance liquid chromatography (HPLC).

(2) To determine if GSH-PGA_1 is the preferred substrate for PGE 9-ketoreductase.

(3) To develop HPLC methods for isolation of specific prostaglandins from urine and plasma for subsequent quantitation by established radioimmunoassay procedures.

Major Findings:

(1) The following method was employed: 500 ml portions of human female urine containing ^3H-PGE_2 tracer, were brought to pH 7.0 and extracted with 200 ml hexane to remove neutral lipids. The aquaeous phase was acidified to pH 3.5 and extracted 3 times with 500 ml $CHCl_3$. The combines organic phases, containing PGE_2 and other acidic lipids, was freed of colloid by filtering through Whatman PS-1 filter paper, dried with $CaSO_4$, and the solvent was removed on a rotary evaporator. The residue was redissolved in 10 ml 90% HOAC and incubated overnight at 70° to convert PGE_2 to PGA_2. PGA_2 was extracted from the acid solution with ethyl acetate and solvent evaporated under a stream of N_2. The residue was dissolved in 0.1 M Tris pH 7.4 containing 10 MM GSH. GSH reacts with PGA_2 to form an inextractable conjugate; other acidic lipids do not react with GSH. After standing for 1 hr at 37°, the solution was acidified to pH 3.5 and extracted with $CHCl_3$ to remove non-reaction lipids. About 30% of the added tracer [H]PGE_2 was also extracted by the ethyl acetate, indicating incomplete conversion to PGA_2 (PGA_1 reacts completely with GSH under the conditions employed). Aqueous phase was brought to pH 13 and allowed to stand for 1 hr at room temperature, during which time GSH-PGA_2 was converted to PGB_2, a form extractable with organic solvents and detectable with the UV-monitor of the chromatograph. The solution was acidified to pH 3.5 and PGB_2 extracted with ethyl acetate. Solvent was removed under a stream of N_2, residue redissolved in a small volume of methanol, and aliquots injected onto a reversed phase column (ODS) eluted with acetonitrile in 0.01% HCOOH. Recovery was about 20% through the procedure. The largest losses occurred during the chromatography step itself and could possibly be reduced with a less adsorptive column. The principal aim of reducing column background to the point that the peak of PGB_2 would not be obscured was only partially achieved, and a preliminary step of open-column or thin layer chromatography would still be required before HPLC analysis could be performed.

(2) Earlier studies of the metabolism of PGA_1 by human red blood cells had revealed two principal products. The first of these, GSH-PGA_1, resulted from conjugation of GSH to the prostaglandin. The second, GSH-9OH-PGA_1, was apparently derived from the first by reduction of the 9-keto group of the prostaglandin moiety. The ability of red cell suspensions and lyzates to convert GSH-PCA_1 to GSH-9OH-PGA_1 was confirmed by subsequent experiments.

Human red cells contain the enzyme prostaglandin E_2-9-ketoreductase, perhaps responsible for this reduction.

With partially purified samples of chicken heart and chicken kidney 9-kR kindly provided by Dr. Lawrence Levine, we found: (2) Both heart and kidney enzymes catalyzed NADPH-dependent reduction of GSH-PGA_1 to GSH-9OH-PGA_1. The identity of the product was confirmed by mass spectrometry. NADPH did not substitute for NADPH as substrate. (b) For both enzymes, GSH-PGA_1 was a much better substrate than PGE_1 or PGE_2. At low substrate concentrations (10^{-8}M) heart enzyme was 24,000 times and kidney enzyme 5400 times more active towards GSH-PGA, than towards PGE_1. At saturating concentrations (10^{-5} M GSH-PGA_1; 10^{-3} M PGE_1) heart enzyme was 20 times and kidney enzyme 7 times more active towards the conjugate. Moreover the km observed for GSH-PGA_1 (2×10^{-6} M, heart enzyme) is 1/50 of that reported for PGE_2. (c) 9-KR exhibited a marked preference for GSH-PGA_1 over L-cysteine-PGA_1 or mercapto-ethanolamine-PGA_1, demonstrating specificity for the ligand attached to the 11-carbon of the prostaglandin moiety. (d) 10^{-5} M PGE_2 weakly inhibited reduction of 10^{-5} M GSH-PGA_1 (17%) and more strongly inhibited reduction of 10^{-8} M GSH-PGA_1 (91%). The reciprocal experiment could not be performed since in an equimolar mixture of PGE_2 and GSH-PGA_1, the conjugate is entirely converted to GSH-9OH-PGA_1 before reduction of PGE_2 is measurable. GSH-9OH-PGA_1 (10^{-5} M) itself inhibited reduction of PGE_2 (10^{-5} M) by 69%.

(3) HPLC is capable of resolving prostaglandins with speed (<0.5 hr) not possible with conventional chromatographic techniques. Recoveries of prostaglandins from Zorbax ODS ranged from 60% for a 0.3 ng infection to 88% when 42 ng was injected. This recovery can be improved to >85% for a 0.2-0.3 ng injection if arachidonic acid (10 ng/ml) is added to the buffer. Addition of arachidonic acid interferes with the radioimmunoassay (RIA).

Before the method can be of value for preparing samples for analysis by RIA, a high and reproducible yield of the prostaglandin must be obtained from the ODS column.

Significance to Biomedical Research and the Program of the Institute: PGA_1 is a potent vasodilator and natriuretic substance which has been used experimentally in the treatment of hypertension and may also be a naturally occurring renal hormone. Understanding the metabolism of PGA_1 may permit the development of more useful drugs which would be less rapidly cleared from the circulation.

The measurement of PGA_1 and other prostaglandins in mammalian tissue presents serious problems due to the extremely low concentrations normally present. Laboratories engaged in prostaglandin measurement by RIA are troubled by the nonspecificity of such assays. If low recoveries can be corrected, HPLC can serve as a highly efficient purification step eliminating the need for specificity in the RIA.

Publications:

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gates of Prostaglandin A_1 in Human Red Blood Cells. J. Biol. Chem. 251: 6550-6554, 1976.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01018-20 LC

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Biochemistry of the Kallikrein-Kininogen-Kinin System

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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)

High molecular weight (HMW) kininogen (Flaujeac, Williams, Fitzgerald, or contact activation factor) is required in conjunction with plasma prokallikrein for the normal rate of activation of surface-bound unactivated Hageman factor.

Turnover studies in rhesus monkeys with ^{125}I-labelled human and monkey low molecular weight (LMW) and HMW kininogens purified by immunoaffinity and DEAE-cellulose chromatography showed similar half-life values but significantly different fractional catabolic rates.

Human plasma prokallikrein has been highly purified in five steps. Isoelectric focusing revealed four major peaks with pI's of 8.5, 8.8, 9.1, and 9.3, besides four minor variants.

Human renal prokallikrein (inactive urinary kallikrein) has been partially purified and characterized. Its molecular weight is ~50,000, as compared with 35,000 for renal kallikrein (active urinary kallikrein). Trypsin-activated prokallikrein is indistinguishable from renal kallikrein with respect to kininogenase activity.

Objectives: Purification of glandular kallikreins and prokallikreins and components of the plasma kinin, clotting and fibrinolytic systems for purposes of characterization and production of specific antiserums. Preparation of purified specific antibodies 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 proteins, affinity adsorbents, and assay methods to studies of human disease states, such as hypertension.

Major Findings: 1) Human Plasma Kininogen. a) Immunology. Immunication of a sheep with kininogen B2α, the major kininogen form isolated and the one with the highest specific activity (Project Numbers Z01 01018-19 LC, Z01 HL 01944-18 HE, and Serial No. NHLI-284--Annual Report for 1974), gave a high-titer antikininogen antiserum which produced a single precipitin line with the immunogen in Ouchterlony double diffusion, but gave two strong lines with plasma or Prep A. The antibody impurity was identified as antiplasmin(ogen). Prep B and kininogens B1-B4 applied to Ouchterlony wells at concentrations 20 to 50 times that required to give a strong precipitin line with antikininogen antibody gave a single, sharp line with rabbit antiplasminogen antiserum or with the sheep anti-B2α antiserum after removing the antikininogen with a B1,2-agarose column. Apparently, the antikininogen-agarose column used to obtain Prep B from Prep A contained a small amount of antiplasminogen, inasmuch as Prep B kininogens derived from Prep A adsorbed to a monospecific antikininogen-agarose column contained no detectable plasmin(ogen) antigen. The monospecific antikininogen antibody was described previously (Z01 HL 01018-19 LC, pp. 411-412). Chromatography of Prep B and its subfractions on lysine-agarose columns removed all detectable traces of plasmin(ogen) antigen. The trace of antigen observed in highly purified kininogen fractions may represent a complex in which plasmin is strongly attached to the carboxy-terminal lysine residue exposed after the release of bradykinin from kininogen either by plasmin itself or by plasma kallikrein. This possibility is credible in view of the known high affinity of plasmin(ogen) for 6-aminocaproic acid and its analog, N-α-substituted lysine, and of plasmin's low k_{cat}/K_m value of 650 sec^{-1} mol^{-1} with kininogen B2α, as compared with the k_{cat}/K_m values of 5×10^5, 2.3×10^4, 2.9×10^4, and 1.4×10^5 found with human renal kallikrein, human plasma kallikrein, bovine trypsin, and porcine pepsin, respectively. Thus, kininogens may act as plasmin inhibitors under certein conditions.

b) Hageman factor cofactor activity of HMW human kininogen B4γ. Using purified proteins (B4γ, plasma prokallikrein, and unactivated Hageman factor), we found that HMW kininogen and prokallikrein are required together for the normal rate of activation of surface-bound unactivated Hageman factor (Factor XII). Factor XIIa (active Hageman factor) was measured by its activation of prokallikrein and Factor XI. Neither HMW kininogen nor prokallikrein is involved in the binding of Factor XII to a surface.

c) Turnover studies. Purified, ^{125}I-labelled human and monkey LMW and HMW kininogens were injected into healthy adult rhesus monkeys to study normal metabloic turnover. The kininogens were purified using immunoaffinity chromatography and further separated into LMW and HMW fractions by DEAE-cellulose chromatography. Blood samples were drawn at various intervals and assayed in

a gamma counter. The disappearance of injected ^{125}I-kininogen from the intravascular compartment could be expressed as a double exponential equation. There was no significant difference between the turnovers of human and monkey kininogens, which also show complete immunological identity by Ouchterlony double diffusion. The difference between the half-life of HMW kininogen (26.0 ± 1.6 h) and that of LMW kininogen (20.2 ± 2.1 h) was only marginal ($P>0.05$). In contrast, there was a highly significant difference in their fractional catabolic rates (1.12 ± 0.01 day^{-1} for HMW kininogen and 2.05 ± 0.12 day^{-1} for LMW kininogen, with $P<0.001$). These differences between the two kininogens are attributed to differences in their distribution between the intra- and extravascular pools.

2) Human Plasma Prokallikrein. a) Purification. The previously described scheme (Serial Nos. NHLI-214 and NHLI-77) for purifying plasma prokallikrein has been modified and extended. The starting material, instead of ACD or CPD plasma, was obtained from the American Red Cross Blood Research Lab, Bethesda, as a by-product of the large-scale purification of coagulation Factors IX and X. Fresh CPD plasma (or frozen fresh plasma) was stirred with preswollen DEAE-Sephadex A-50 (1.5 g dry weight/liter of plasma) at pH 7.2 and ~25° for 0.5 to 1.0 h. The filtrate, containing about 95% of the starting plasma proteins and all of the starting prokallikrein activity, was obtained the same day and dialyzed three times at ~25° against 10 volumes of 0.01 M Tris-HCl/0.01% polybrene/0.1% NaN_3, pH 7.0. The supernate from the centrifuged sac contents was chromatographed at ~25° on a column of DEAE-cellulose (DE 52) previously equilibrated with the dialysis buffer. This step removes all of the acidic plasma proteins, including Hageman factor (which when activated converts plasma prokallikrein to kallikrein), plasminogen, kininogen, etc., but allows all of the prokallikrein to pass into the colorless filtrate. As before, the proenzyme was adsorbed to an agarose-dodecanediamine-X-L-arginine column, but at 4° instead of the previous 25°. Elution of the thoroughly washed column with 20 mM acetic acid (~pH 3.2) gave a 25-fold purification of prokallikrein in 95-100% yield. Further purification was achieved by CM-Sephadex C-50 and CM-cellulose chromatography, Sephadex G-150 gel filtration, and isoelectric focusing (all at 4°). The last step gave four forms of highly purified prokallikrain with pI's of 8.5, 8.8, 9.1, and 9.3, as well as four minor forms with pI's of 7.9, 8.2, 9.4, and 9.6.

b) Stability. Prokallikrein in the DEAE-cellulose filtrate (made to 0.1% NaN_3) was stable for several days at pH 5.0 (4° and 25°) and pH 7.9 (4°). However, at pH 7.0 and 25°, activation was complete within a few days. Purified prokallikrein was stable at pH 5.0 (4° and 25°) for several days, but was gradually activated at pH 7.9 (4° and 25°).

3) Human renal kallikrein and prokallikrein (previously called free and bound or active and inactive urinary kallikreins, respectively). As reported in Project No. Z01 HL 01016-06 LC, fresh human urine contains between 25 and 75% of its kallikrein in an inactive form which can be activated by bovine trypsin. Activation also occurs spontaneously, especially in purified and concentrated fractions. Urine and 100- to 1,000-fold concentrates of urine obtained by ultrafiltration retain their inactive kallikrein for several weeks

or months at 4-6° in the presence of 0.02-0.1% NaN_3. The urinary kallikrein formed by trypsin activation is indistinguishable from the naturally occurring enzyme in its ability to form kinin from heated human plasma or from highly purified human kininogen B2α. Inactive kallikrein has been partially purified from urine at 4-5° by: (1) concentration in an Amicon hollow fiber apparatus; (2) precipitation of the Tamm and Horsfall glycoprotein with 0.2 M NaCl, followed by centrifugation; (3) adsorption of the dialyzed supernate to a DEAE-cellulose column equilibrated with 0.01 M NaP_i, pH 6.0, followed by a wash of 0.01 M NaP_i, pH 6.0, and a linear gradient of 0.10 to 0.35 M NaP_i, pH 6.0; and (4) Sephadex G-100 gel filtration of the inactive part of the partially overlapping active and inactive kallikreins. Molecular weight estimates of ~38,000 and ~50,000, respectively, were obtained by gel filtration of the DEAE-cellulose fraction on a column of Ultrogel Ac-A44 in 1 M NaCl, when compared with molecular weight markers (bovine serum albumin, ovalbumin, hog pancreatic kallikrein, and myoglobin). All of the inactive kallikrein preparations obtained above inhibited trypsin during the activation step of the ^{3}H-TAME assay. Thus, the data favored the hypothesis that inactive kallikrein is an enzyme-inhibitor complex. However, inactive kallikrein was not activated during gel filtration of the DEAE-cellulose fraction in 6 M guanidine-HCl on an agarose (Bio-Gel A-15m) column--i.e., there was no separation of inhibitor (estimated molecular weight of 15,000) and active enzyme (35,000 molecular weight). Furthermore, hydroxyapatite chromatography of the DEAE-cellulose fraction, using a linear gradient of 0.01 to 0.10 M NaP_i, pH 6.0, in the presence of 3 M NaCl at 4°, gave a peak of inactive kallikrein free of the trypsin inhibitor, as well as being almost completely separated from the active form. Two different regions of the hydroxyapatite chromatogram contained trypsin inhibitor. Thus, the evidence now strongly supports the hypothesis that inactive urinary kallikrein is a proenzyme.

Significance to Biomedical Research and the Program of the Institute: Highly purified components of the plasma kallikrein-kinin system are crucial to investigations of its physiological function. This system is activated simultaneously with the intrinsic blood coagulation and fibrinolytic systems. Besides plasma prokallikrein, HMW kininogen also is required for the normal rate of surface activation of Hageman factor (Factor XII). Active Hageman factor (XIIa) acts upon its two natural substrates (Factor XI and prokallikrein) to initiate these three systems. Therefore, the chemistry and mechanism of action of pure HMW kininogen should be studied. Similarly, highly purified components of the renal (urinary) kallikrein-kinin system will be required to study its role in the kidney.

Proposed Course of Project: Kininogen. We plan to compare kininogen turnover with that of fibronogen and plasminogen in infected monkeys with disseminated intravascular coagulation. Attempts to separate kininogen types I and II by isoelectric focusing and other methods will be made.

Plasma prokallikrein. We plan to purify the several forms of prokallikrein to homogeneity for various physicochemical and biochemical studies.

Renal prokallikrein. Further attempts will be made to purify this proenzyme and to seek means of preventing its activation during purification.

Publications:

Meier, H.L., Scott, C.F., Mandle, R., Webster, M.E., Pierce, J.V., Colman, R.W. and Kaplan, A.P.: Requirements for contact activation of human Hageman factor. Ann. N.Y. Acad. Sci. 283: 93-103, 1977.

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PROJECT NUMBER (Do NOT use this space) | DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | PROJECT NUMBER Z01 HL 01019-01 LC

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Purification and Characterization of Kininogen in Human Urine

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

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Section on Physiological Chemistry

INSTITUTE AND LOCATION

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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)

To understand the role of the kallikrein-kinin system in the kidney it is necessary to know where kinins are formed. Recently it was shown that kallikrein arises from the distal tubule. We have found kininogen in the tubule and in urine and believe that kinins are formed in the tubule. Methods are under development to assay, characterize and determine the significance of urokininogen.

PHS-6040
(Rev. 10-76)

Objectives: To develop methods for the purification and characterization of kininogen in human urine. To establish the role of urinary kininogen-kallikrein-kinin system in health and disease.

Methods Employed: Radioimmunoassay of kininogens and kinin (prog #Z01 HL 01016-07 LC). Protein purification and characterization using ion-exchange and affinity chromatography.

Major Findings: Urine from five normal subjects was analyzed for kininogen content. Kininogen was determined by radioimmunoassay of kinin before and after tryptic digestion of heated acidic urine. Kinin generated by trypsin is released from kininogen. In urine from one male and two females no kininogen was detected. Urine samples from two males contained 3.0 and 13.8 ng kininogen per ml, with increases in kinin levels of 35% and 47% respectively. On three occasions, a large volume of pooled urine from normal males was collected in the presence of sodium azide and analyzed immediately. No kininogen could be detected in any of these pooled samples.

The stability of kininogen was examined in urine collected and stored under different conditions. No consistent differences could be found among samples collected in the presence of hydrochloric acid and pepstatin, with sodium azide or without preservatives. In one sample without preservatives over half of the kininogen was found three days later. In another sample containing sodium azide, more than two-thirds of the kininogen was present after fifteen days of storage at 4°C.

Pooled urine samples were dialyzed against 0.01 M sodium phosphate pH 6.3 and stored in the cold at pH 6.3 or pH 2.0. These samples were compared with non-dialyzed samples collected and stored under similar conditions. The dialyzed sample at pH 6.3 contained 0.5 ng/ml kininogen, while no kininogen could be detected in the non-dialyzed urine. Approximately 1.5 ng/ml kininogen were measured in the dialyzed sample stored at pH 2, a quantity which was nearly twice that of the non-dialyzed sample. After 24 hours of storage at 4°C, no kininogen could be detected in the non-dialyzed acidic urine.

In addition to the effects of dialysis and storage conditions, kininogen content was greatly influenced by the quantity of trypsin employed. In the non-dialyzed acidic sample, kinin generation could be measured only when large quantities of trypsin (200 µg/ml urine) were employed. Kinin could be generated in the dialyzed smaples, however, with as little as 2 µg/ml urine, although twice as much kinin was formed when the amount of trypsin was increased to 200 µg/ml.

Ion exchange chromatography has been applied successfully to purification of plasma kininogen in our laboratory (prog. #Z01 HL 01018-19 LC). In preliminary experiments, urinary kininogen did not adsorb to DEAE-cellulose, DEAE-Sephadex, or QAE-Sephadex at pH 6.2. Further investigation will be required to determine the conditions necessary for chromatography of urinary kininogen.

Significance to Biomedical Research and the Program of the Institute: Purification and characterization of urinary kininogen is essential to the

evaluation of the pathophysiological significance of the renal kallikrein-kinin system.

Proposed Course of Project: To continue current investigations on the purification and characterization of urinary kininogen.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01020-01 LC

PERIOD COVERED

July 1, 1976 to May 31, 1977.

TITLE OF PROJECT (80 characters or less)

Determinants of Hormone Action

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)

Vasopressin, Ca^{++}, carbechol, theophylline and ouabain failed to alter the rate of phospholipid turnover in rat renal cortical slices.

Ranatensin markedly inhibited sodium transport in the toad bladder. The inhibition was not affected by atropine, but was blocked by pretreatment of submaximal doses of ouabain.

PHS-6040
(Rev. 10-76)

Objectives: To study the physiologic and biochemical determinants of hormone action at the level of the renal tubular cell. Specifically:

1. The roles of phospholipid-hormone interaction and hormonal control of phospholipid turnover on transport processes.

2. The effects of vasoactive peptides on sodium transport.

Methods Employed:

1. Rates of phospholipid turnover were determined after appropriate incubation of rat renal cortical slices or toad bladder segments with [^{3}H]-inositol, [^{14}C]-choline and [^{14}C]-ethanolamine, precursors of the respective phospholipids. Classes of phospholipids were extracted and separated by TLC. The effects of various hormonal agents (ADH, carbechol, cAMP, ouabain, Ca^{++}, theophylline) were then studied on the rate of incorporation of radiolabeled inositol, ethanolamine, and choline into phospholipid and the rate of release of these compounds from previously labeled phospholipids.

2. The effects on sodium transport of the vasoactive peptides, neurotensin, substance P, ranatensin, enkephalin, and endorphin were studied in the toad bladder using a standard Ussing apparatus.

Major Findings:

1. Rapid incorporation of labeled precursors into phospholipid occurred in rat cortical slices. In toad bladder segments, incorporation of [^{3}H]-inositol into phosphotidylinositol occurred at approximately 10% of the rate of incorporation into mammalian cells. Extensive testing with vasopressin, Ca^{++}, carbechol theophylline and ouabain failed to alter the rate of incorporation or release of water soluble products from preformed phospholipid. Using the methods employed, it appears the above agents do not directly affect phospholipid turnover.

2. Studies with ranatensin on short circuit current in the toad bladder demonstrate a marked inhibition of sodium transport unaffected by atropine. Furthermore, its inhibitory action is blocked by pretreatment with submaximal doses of ouabain suggesting a similar site of action of these two agents.

Significance to Biomedical Research and the Program of the Institute: The control of phospholipid turnover may be an important cell transport regulatory mechanism. Numerous studies involving phospholipase activation. and cholinergic phospholipid receptors suggest a potentially important role of phospholipids in cell function and possibly hormone membrane interaction regulating transport events.

The occurrence of a naturally occurring peptide that inhibits sodium transport and is blocked by ouabain suggests the possibility of a naturally occurring negative control mechanism operating through a glycoside binding site in the amphibian.

Proposed Course:

1. Further studies are planned to investigate the role of naturally occurring phospholipases in the toad bladder and the role of aldosterone.

2. The nature of the inhibitory action of ranatensin on short circuit current is being pursued utilizing [^{22}Na] flux studies and analogues of ranatensin to assess its active site.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01021-01 CH

PERIOD COVERED

July 1, 1976 to May 31, 1977

TITLE OF PROJECT (80 characters or less)

Enzymatic Studies on Cultured Human Vascular and Smooth Muscle Cells: Angiotensin and Kinin Systems

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

Laboratory of Chemistry

SECTION

Section on Physiological Chemistry

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

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)

Human endothelial cells (HEC) and smooth muscle cells (SMC) were incubated with angiotensin I (AI), bradykinin, angiotensin II (AII), tetradecapeptide renin substrate and hog plasma renin substrate to observe antiotensin converting enzyme (ACE), kininase, angiotensinase and renin activities. AI conversion, disappearance of kinin and AII, and generation of angiotensin from renin substrates were measured by bioassay on rat uterus and/or guinea pig ileum. Angiotensin conversion also was studied using [^{3}H]-(10-Leu)-AI and monitoring disappearance of radioactive AI and appearance of [^{3}H]-His-Leu by electrophoresis on cellulose plates. The product of incubation of HEC with [^{3}H]-(8-Phe)-bradykinin was shown to be ^{3}H-Phe-Arg by co-chromatography with authentic Phe-Arg. The molecular weight of ACE was estimated by gel filtration and by sucrose density gradient centrifugation.

Major Findings: Human vascular endothelial and smooth muscle cells were selectively cultured from term umbilical cord veins and examined for angiotensin converting enzyme (kininase II), angiotensinase, and renin. One peak of angiotensin converting activity was observed in gel filtrates of mechanically harvested endothelial cells. This peak closely resembles the highly purified enzyme from lung and kidney. It releases histidylleucine from angiotensin I and phenylalanalylarginine from bradykinin, requires Cl for maximal activity, is partially inhibited by ethylenediamine-tetraacetic acid and strongly inhibited by the snake venom nonapeptide Pyr-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro. Its molecular weight, estimated by gel filtration, was 140,000; by sucrose density gradient centrifugation, 115,000. No angiotensin converting activity was found in conditioned medium from either endothelial or smooth muscle cell cultures, nor in sonicates of smooth muscle cells. Antiotensinase activity was not detected in endothelial cell sonicates at pH 7.0 or 7.4, but substantial activity was observed at pH 5.8. Endothelial cell sonicates also actively generated angiotensin I from the tetradecapeptide renin substrate at pH 5.0 or 5.6. However, this enzyme appears to be different from renin and pseudorenin in that it was not inhibited by pepstatin and retained some activity at pH 7.5. These data provide further evidence for an active role of endothelium in the local control of vascular tone.

Significance to Biomedical Research and the Program of the Institute: These findings, together with other data, point to a central role for the endothelial cell in the response of blood vessels to vasoactive peptides. Formation of a potent vasoconstrictor, AII, and degradation of a potent vasodilator, bradykinin, both appear to occur close to the vessel wall-blood interphase, through the action of endothelial CE (Kininase II).

The demonstration of CE and renin-like activity in an extrapulmonary endothelium supports the concept that the renin-angiotensin system can function at a local level in modulating vascular tone. Vascular CE activity would contribute to the pressor activity of any AI generated in arterial blood, as well as that which bypasses the pulmonary circuit due to anatomical shunting. The further presence of a renin-like activity in cells of the vessel wall could permit direct, local generation of AI from circulating renin substrate. Intramurally produced AII could act as a short-range hormone, with ready access to target cells in the vessel wall. The effective concentration of agonist in the vicinity of physiological receptors, therefore, might be several orders of magnitude greater than that detectable in the peripheral circulation. The frequent lack of correlation between clinically apparent hypertensive disease and peripheral plasma renin activity or AII concentrations might be explained on this basis. Furthermore, locally generated AII would influence the number of free receptors available to circulating AII. Thus, physiological factors which modify the activities of endothelial converting and renin-like enzymes could be important determinants of the intrinsic tone and reactivity of peripheral vessels.

Proposed Course of Project: Further research is not planned.

Publications: None

ANNUAL REPORT OF THE
LABORATORY OF KIDNEY AND ELECTROLYTE METABOLISM
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
July 1, 1976 through Sept. 30, 1977

The continuing goal of research in this laboratory is to elucidate the mechanisms of salt and water homeostasis in the kidney and other tissues in order to establish a basis for understanding the normal function as well as the pathophysiology and treatment of disease processes. In order to achieve the goal we utilize a wide range of techniques, many of which have originated within our laboratory.

Isolated segments of renal tubules

Current advances in kidney physiology rely heavily on the direct study of individual nephron segments. The intact organ is too complex to be understood without analysis of its individual parts. One widely used method which originated in this laboratory is to dissect individual tubules and perfuse them in vitro. As detailed in previous reports, direct study of nephron segments in this manner has revealed a surprising diversity of function and uncovered a number of unexpected processes.

Currently, a variety of transport processes in different nephron segments are being studied:

1. Transport of bicarbonate by collecting tubules. The kidneys are the major regulators of body acid-base balance. Control is achieved by the renal tubules which transport bicarbonate and/or hydrogen ions. Last year Drs. McKinney and Burg reported that proximal tubules perfused in vitro absorb bicarbonate from the tubule fluid and that the bicarbonate absorption by that segment is heavily dependent on sodium. This year they studied bicarbonate transport by collecting tubules. There were two major findings: 1) the direction of bicarbonate transport by collecting tubules in vitro depended on the state of the animal from which they were removed. Tubules from acidotic rabbits absorbed bicarbonate from the tubule fluid; those from alkalotic rabbits secreted bicarbonate into the tubule fluid. Thus, the tubules perfused under standard conditions in vitro continued to respond in a manner appropriate for homeostasis in the living animal. A likely explanation is that there is some enzyme induced as part of the control mechanism, and the investigators are attempting to identify the enzyme. Bicarbonate secretion by renal tubules had not been previously reported

and may represent an important part of the adaptation to alkalosis. 2) Bicarbonate absorption (in the tubules from acidotic animals) was not dependent on sodium as it was in proximal tubules. Thus, the mechanism of acidification by collecting tubules is basically different from that in proximal tubules. An important part of future efforts will be devoted to more detailed analysis the transport mechanisms and their control in various nephron segments.

2. Transport of calcium and other ions by thick ascending limbs of Henle's loop. Studies of ion transport by renal tubules is limited by the small size of the samples available. Elemental analysis of such small samples is possible, however, using the electron probe. Drs. Bourdeau and Burg have been working for the past year to adapt this method to study calcium, magnesium, and phosphate transport by renal tubules. Unfortunately, because no electron probe is available at NIH, they have had to send samples to Boston for analysis. Although the results are promising, the method is not yet perfected. Meanwhile, they are using radioisotopes to study calcium transport by the thick ascending limbs. The important finding is that the absorption of calcium by this segment appears to be passive, driven by the transepithelial voltage, as is the transport of sodium and potassium. The observation explains why diuretic drugs such as furosemide which reduce the voltage in this segment cause urinary calcium excretion to increase.

3. Control of sodium and potassium transport by collecting tubules. It has been proposed that collecting tubules which are the terminal nephron segments may control urinary sodium and potassium excretion, but it has been difficult to confirm this theory or to elucidate the mechanisms involved. One possible mechanism is that the tubules could change intrinsically due to induction of an enzyme. This effect could be identified by changes in function of the tubules in vitro after they are removed from the animal. To test this possibility Drs. Schwartz and Burg gave a variety of diets and drugs to rabbits to alter urinary sodium and potassium excretion and then studied their renal tubules under standard conditions in vitro. The question was whether homeostasis involved changes in function which persisted after the tubules were isolated from the animals. They found such changes under some conditions. For example, after the rabbits were given a low salt diet for several days, the rates of sodium absorption and potassium secretion by the tubules increased in vitro. On the other hand, after two days of severe salt depletion produced by diuretics, there was no change in the function of the isolated tubules despite the fact that urinary salt excretion was greatly diminished. The change in tubule function correlated with plasma aldosterone level which was elevated after the chronic

low salt diet, but not elevated after acute salt depletion. On the other hand, after a high sodium, low potassium diet had been given to the rabbits, the tubules in vitro transported both sodium and potassium less rapidly. In this case no plasma aldosterone was detectable. Transport was not restored, however, by adding aldosterone in vitro, so it is uncertain what role lack of aldosterone had in this response.

Necturus proximal tubule and gall bladder:

Epithelia, including kidney tubules, are complex structures containing a variety of membranes and channels. Understanding the function of the epithelia requires that the structures involved in transport be identified and that individual transport steps be characterized. Dr. Spring is applying sophisticated electrophysiological, optical, radioisotopic and mathematical techniques to these problems. He has been investigating transport in two epithelia:

1. Chloride transport by renal proximal tubules of Necturus. Drs. Spring and Kimura have used chloride sensitive microelectrodes to determine that the entry of chloride into proximal tubule cells from the tubule lumen involves an interaction with the luminal membrane. Necturus proximal tubule cells were found to take up chloride only when NaCl was present in the lumen, not when other salts of chloride were present. The cellular uptake process is electrically neutral. The investigators believe that the entry of chloride into the cells is coupled to the entry of sodium and that energy from the downhill movement of sodium into the cells drives chloride into the cells uphill, resulting in chloride activity greater in the cells than in the extra cellular fluids. The entry of chloride into the tubule cell is against the electrochemical gradient for that ion but the exit from the cell to the blood is downhill and may be completely passive. They estimated that 40-50% of the chloride normally reabsorbed by the proximal tubule is transported across the cellular pathway. The remainder diffuses out of the tubule lumen through a shunt pathway between the epithelial cells. Studies with radioactive chloride are presently underway to precisely quantitate the movements of that ion across the epithelium through the various pathways.

2. Role of the lateral intercellular spaces in transport by the Necturus gallbladder. Drs. Spring and Hope are studying the role of the lateral intercellular spaces of the Necturus gallbladder in determining the functional properties of the epithelium. Through the use of a specially designed tissue chamber and microscope they have been able to visualize the lateral intercellular spaces (which are extremely small in the living tissue) and quantitatively measure their size

and shape. They are presently determining the relationship between hydrostatic pressure and lateral intercellular space volume. These experiments should lead to resolution of several questions about the mechanism of fluid transport and its control in gallbladder and other leaky epithelia. More realistic models of fluid transporting epithelia await values for parameters being measured in these experiments.

Toad urinary bladder

Toad urinary bladder epithelium has been used extensively as a model salt and water transporting system, resembling in this respect the distal nephron. It has been used in this laboratory to study vasopressin and aldosterone control of salt and water excretion.

During the past year, Dr. Handler together with Drs. Zussman and Keiser of the Hypertension-Endocrinology Branch demonstrated that the toad bladder in vitro produces prostaglandin E (PGE) and that the rate of production of PGE is increased by vasopressin. It was previously known that exogenous PGE inhibits the action of vasopressin on water permeability. Thus stimulation of PGE production by vasopressin serves to modulate the effect of the hormone. In further studies they found that chlorpropamide, (which is used in the treatment of pituitary diabetes insipidus) enhanced the effect of vasopressin by inhibiting PGE synthesis. Adrenal steroid hormones also enhance the response of the toad bladder to vasopressin. This effect was previously explained (at least in part) by reduction in the activity of cyclic nucleotide phosphodiesterase, the enzyme that catalyses the degradation of cyclic AMP. The investigators have now found that adrenal steroid hormones also reduced the rate of PGE synthesis, contributing to the enhancement of the response to vasopressin. The adrenal steroids inhibited PGE biosynthesis by inhibiting a phospholipase (acylhydrolase) that catalyses the release of arachidonic acid, a precursor of PGE, from storage in tissue lipids. Therefore, PGE produced in the toad bladder is not only an endogenous inhibitor of the response to vasopressin, but is regulated by other hormones, mediating their effect on the response to vasopressin.

It was previously shown that catecholamines with alpha adrenergic activity inhibited the water permeability response of the toadbladder to vasopressin by inhibiting adenylate cyclase, the enzyme that catalyses the formation of cyclic AMP. This year Drs. Handler and Sherman found that under special conditions alpha adrenergic drugs also had a second effect which was to stimulate rather than inhibit vasopressin activity. Toad bladders were incubated with high concentrations

of norepinephrine. After the catecholamine had been removed the tissue was more responsive to vasopressin than paired tissue that had not been incubated with norepinephrine. This second effect was blocked by phentolamine, an alpha adrenergic blocking agent, indicating that it also is alpha adrenergic. The mechanism of the stimulation remains to be determined.

Dr. Handler noted that toad bladders incubated with high concentrations of vasopressin for a prolonged period became refractory to the hormone and only gradually recovered sensitivity when incubated in vasopressin-free media. Neither the refractoriness nor recovery are dependent upon protein synthesis, since they were uneffected by cyclohexamide or actimomycin-D. The mechanism of refractoriness is also under continued investigation.

Cyclic 3',5' guanosine monophosphate (cyclic GMP) affects the response of some tissues to hormones by antagonizing the action of cyclic AMP. Cyclic GMP is present in kidneys and in urine but its role is unknown. In order to investigate its role Drs. Sahib and Handler measured its concentration in toad bladder epithelial cells. The concentration of cyclic GMP was two orders of magnitude lower than the concentration of cyclic AMP. Carbamyl choline caused the cyclic GMP level to increase and inhibited sodium transport (opposite to the effect of cyclic AMP which enhances sodium transport). These results are consistent with the theory that cyclic GMP antagonizes the action of cyclic AMP on sodium transport in the toad bladder. Further investigation of the relationship has been limited, however, by inability to find other agents which cause cyclic GMP and cyclic AMP to vary independently and by the lack of any effect of exogenous cyclic GMP on sodium transport in the toad bladders.

Cyclic nucleotide phosphodiesterases in rat kidney

The only known degradative pathway for 3',5' cyclic nucleotides is hydrolysis to the corresponding 5' nucleotides. This step is catalysed by cyclic nucleotide phosphodiesterases. Cellular cyclic nucleotide levels are regulated by the balance of phosphodiesterases which degrade them and purine cyclase which synthesizes them. Drs. Strewler and Orloff, in collaboration with Drs. Vaughan and Manganiello of the Laboratory of Cellular Metabolism have studied the control of phosphodiesterase in the kidney. Treatment of rats for 3 days with the glucocorticoid hormone dexamethasone resulted in decreased cyclic GMP phosphodiesterase activity in renal cortex. This is similar to the effect of glucocorticoids on

phosphodiesterase in other tissues. In the course of these studies it was observed that cyclic AMP phosphodiesterase activity increased spontaneously with time in homogenates of rat renal cortex. This observation led to the identification of an endogenous protein activator of phosphodiesterase. The activator is associated with a particulate fraction of renal cortex. It differs from previously described phosphodiesterase activators in that it does not require calcium and is heat labile. Its action results in a change in the sedimentation properties of phosphodiesterase consistent with a decrease in molecular weight, which favors the interpretation that the activator is a protease. Studies are currently underway to purify the activator and to assess its possible role in hormone-induced increases in phosphodiesterase activity in kidney and other tissues.

Molecular mechanisms of transport in membranes of human red blood cells

Dr. Cabantchik, who is new to this laboratory, is investigating the relation of specific membrane proteins to the transport of anions and nucleosides by human red blood cells. This represents a continuation of work that he started elsewhere. He uses chemical probes that interact specifically with the transport systems to label the membrane proteins involved, so that he can identify, characterize, isolate, and reconstitute them.

In his previous work Dr. Cabantchik identified the anion exchange system of the erythrocyte as "band 3," a 95,000 dalton protein. He incorporated purified band 3 protein into phospholipid vesicles, reconstituting the anion transport system. The purified vesicle system has several interesting properties that he is now investigating: 1) the energy of activation of the system is governed by the lipid composition of the medium. 2) Anion transport by the vesicles is not affected by pH below 6.3, whereas transport by native red cell membranes is. The pH effect in the native membranes may involve another membrane protein which is lacking in the purified system. If so, it should be possible to characterize the interaction of the proteins. 3) Inhibitors act asymetrically on the anion transport system whereas the anion exchange is symmetrical.

Future sudies will involve coupling of flourescent probes to specific sites on the band 3 protein, incorporation of the labeled protein into vesicles, and measurement of dynamic spectral changes in flourescence that result from addition of substrates and inhibitors, or changes in pH and temperature. Also, it is known that the 20,000 dalton fragment of the band 3 protein that normally lies within the lipid of the membrane is itself sufficient to maintain anion exchange. This fragment will be isolated and characterized.

Dr. Cabantchik previously showed that the nucleoside transport system of human erythrocytes consistently behaves kinetically as a simple carrier when tested in a number of different ways. No other transport system that has been examined proved to be a simple carrier by all of these criteria. He intends to identify, isolate, and reconstitute the nucleoside carrier as he did the anion exchange and compare the properties of this nucleoside system which is electrically neutral to those of the anion system which is electrically charged. He has already designed and synthesized photoaffinity probes for the nucleoside system. These chemicals interact reversibly with the transport system in the dark, but when activated by light, bind tightly to the receptor sites. The use of radioactive photoaffinity probes will facilitate identification and isolation of the transport proteins. He also has coupled chemicals with a high affinity for the transport system to an isoluble matrix (Sephadex beads) for use in isolating the transport system from red cell membranes.

Control of volume of nucleated red blood cells

Cells from a number of tissues regulate their volume by taking up or losing potassium and to a lesser extent sodium. The cation movements are accompanied by movements of anions, followed by water. Dr. Kregenow performed much of the original work which identified this phenomenon, utilizing nucleated avian erythrocytes as a model system. He reported in previous years that the active sodium and potassium transport system of red cells is not involved in the process. Instead, the regulation of cell volume involves large increases in cation permeability which facilitate cation movement. The net transport of the cations under these conditions generally is passive down their electrochemical gradients.

This year Dr. Kregenow observed that the regulation of cell volume was accompanied by production or consumption of large amounts of acid. During cell enlargement acid was produced, causing the pH of the medium to decrease. During cell shrinkage the opposite occurred. The amount of acid produced or consumed correlated with net cation and water movement. The anion from the acid did not appear in the medium and its identify remains unknown. The role of the acid in cell volume homeostasis also is unknown and is an important problem for future investigation.

Norepinephrine is a hormone that initiates cell enlargement by the mechanism discussed above. Dr. Kregenow is studying

interaction of the hormone with receptors on the cells. Hormonal recognition can be viewed as a lock (receptor) and key (hormone) phenomenon. He has found that a number of structural analogues of norepinephrine and epinephrine induce cell enlargement, but to different extents. The analogues are derivatives of phenylalanine which is an amino acid. Correlation of the chemical structure of the analogues to their activity will contribute to the understanding of both hormone-receptor and amino acid-receptor interactions.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01214-02 KE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Cyclic nucleotide metabolism in toad urinary bladder

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

PI: M. K. Sahib, Ph.D., Visiting Scientist, LKEM/NHLBI
Other: J. S. Handler, M.D., Head, Section on Membrane Metabolism, LKEM/NHLBI

COOPERATING UNITS (if any)
J. H. Schwartz, M.D., Dept. of Nephrology, WRAIR, WRAMC, Washington, D. C.

LAB/BRANCH
Laboratory of Kidney & Electrolyte Metabolism

SECTION
Section on Membrane Metabolism

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, Bethesda, Md.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	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)

This project is concerned with defining the role of cyclic 3', 5' guanosine monophosphate in the inhibition of sodium transport by carbamyl choline (carbachol) in toad urinary bladder.

Project description:

Objective

Cyclic 3',5'-guanosine monophosphate (cGMP) is found in normal urine and mammalian kidney but its role (if any) in the regulation of kidney function remains to be determined.

Last year (see annual report Z01 HL 01214-01 KE, 1976) we initiated a study of cGMP metabolism in epithelial cells of the toad urinary bladder, a membrane that has transport properties and hormone responsiveness analogous in many respects to the mammalian distal nephron. We found that cGMP levels are two orders of magnitude lower than cAMP levels and that carbamyl choline increases cGMP levels and inhibits sodium transport. The purpose of this study has been to elucidate the role of cGMP in toad bladder epithelial cells.

Methods

Tissue extracts were succinylated and assayed for cAMP and/or cGMP by radioimmunoassay.

Major Findings

10^{-4} M carbachol inhibited short-circuit current (scc) when added to the solution on the serosal side of the bladder. This inhibition began almost immediately and persisted for at least an hour. Peak inhibition was reached at 5 min. Isotopic flux measurements revealed that this inhibition of scc can be totally accounted for by reduction in mucosa to serosa flux of sodium. Peak inhibition of sodium transport was preceded by peak accumulation of intraepithelial cGMP which was maximal at 1 min., the earliest time the measurement could be made. Although maximal stimulation of cGMP occurred in 1 min., 2 min. values were less variable and were used for subsequent studies. 10^{-6} M atropine, a specific muscarinic blocking agent completely blocked both the cGMP and scc responses to carbachol. Dose-response curves of the effect of carbachol on scc and cGMP were similar at low concentrations of carbachol.

Isobutylmethylxanthin (IBMX) a cyclic nucleotide phosphodiesterase inhibitor caused the basal level of both cAMP and cGMP to increase significantly without a significant change in basal scc. IBMX did not affect either the decrease in scc or increase in cGMP in response to carbachol. We suggest that cAMP and cGMP may have opposite effects which cancel when IBMX causes both to increase. The effect of vasopressin was similar to that of IBMX. Carbachol caused the same increase in cGMP whether vasopressin was present or not, but vasopressin markedly diminished

the fall in scc caused by carbachol. We suggest that vasopressin caused cAMP to increase which blunted the inhibition of sodium transport elicited by carbachol.

Recently it was observed (Weisman et al (1977) JCI 59:418) that calcium ionophone A23187 inhibits scc in toad urinary bladder, associated with increased flux of Ca^{+2} into the epithelial cells. We have observed that the ionophore also elevates intraepithelial cGMP. Thus, evidence at hand supports the concept that cGMP mediates inhibition of sodium transport by carbachol.

Significance to Biomedical Research and Program of the Institute

The evidence gathered in this project increases the information about intracellular factors that regulate sodium transport.

Proposed Course

The project is completed.

Publications

Sahib, M. K., J. H. Schwartz, J. S. Handler: Inhibition of sodium transport by carbachol: potential role of cyclic guanosine monophosphate. presented at American Federation For Clinical Research in Washington, D. C. on May 2, 1977. Clin Res. (1977) 447A.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01216-02 KE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Pathways of chloride movement across Necturus proximal tubules

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

PI: Kenneth R. Spring, DMD, Ph.D., Sr. Staff Fellow, LKEM, NHLBI
Other: Genjiro Kimura, M.D., Visiting Associate, LKEM/NHLBI

COOPERATING UNITS (if any)
none

LAB/BRANCH
Laboratory of Kidney and Electrolyte Metabolism

SECTION
Section on Electrolyte Transport

INSTITUTE AND LOCATION
NHLBI/NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1-1/2	1-1/2	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The pathways and mechanisms of transepithelial transport of chloride ions are being investigated in the proximal tubule of the Necturus kidney. Chloride sensitive intracellular micro-electrodes were constructed to monitor intracellular chloride activity and to determine cellular responsiveness to changes in extracellular chloride concentration. Tracer chloride fluxes across the tubule were measured to quantitate the chloride movements across the tubule cells and through the shunt pathway.

PHS-6040

Objectives

Prior to this investigation it was not known whether the reabsorption of chloride by Necturus proximal tubule was a purely passive, extracellular phenomenon or whether the tubule cells were directly involved. We wished to determine the magnitude of chloride movements through tubule cells as well as around them, through the extracellular shunt paths. Measurements of intracellular chloride activity with ion sensitive electrodes can be used to determine the responsiveness of cell chloride to changes in extracellular chloride. Chloride tracer fluxes can also be utilized to further characterize the movements of this ion.

The principal object of this investigation is the quantitation of the fluxes of chloride ions across the cellular and extracellular pathways of Necturus proximal tubules. In addition estimates for cellular membrane chloride permeability as well as extracellular permeability will be obtained.

Methods

Microelectrodes which sense chloride activity and are small enough to be inserted into tubule cells have been developed. Glass electrodes are fabricated utilizing a microfiber glass capillary specially drawn in the NIH glass shop. The capillary is drawn to a tip of less than 1 μm. It is exposed to the vapors of a volatile siliconizing agent (dimethyl-dichlorosilane)for 60 seconds and the electrode baked for one hour at 100°C. After cooling, the siliconized electrode is partially filled with chloride ion exchange resin. Since the chloride sensing electrode has a very high electrical resistance (10^{11} 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 chloride is then made objectively by this computer system.

An isolated perfused Necturus kidney is prepared so that complete control of the capillary composition may be achieved. Recordings of cell membrane potentials and chloride readings are made under control conditions. The tubule lumen is then perfused continuously with a test solution and the measurements repeated.

In the tracer experiments, the tubule lumen contains a large split droplet of labelled solution. This solution contains $Na^{36}Cl$ and ^{3}H-Inulin or TMA ^{36}Cl and ^{3}H-Inulin. The rate of disappearance of ^{36}Cl from this mixture is determined by reaspirating the droplet after varying periods of time. Dual label counting enables determination of both chloride flux and volume changes.

Major Findings

Movement of Cl from the lumen of Necturus proximal tubule into the cells is carrier mediated and dependent on the presence of luminal Na. Intracellular Cl activity was monitored with ion selective microelectrodes; cell Cl activity was 24.5±1.1 mM in Cl Ringer perfused kidneys. Cell Cl activity is 2 to 3 times that predicted for passive distribution. When luminal NaCl was partially replaced by mannitol (capillaries perfused with normal Cl Ringer) cell Cl decreased showing a sigmoidal dependence on luminal NaCl. Peritubular membrane potential was unaltered. Na sulfate Ringer perfusion of the kidneys washed out all cell Cl but did not alter peritubular membrane potential. Chloride did not enter the cell when the lumen was perfused with 100 mM KCl, LiCl or tetramethyl ammonium Cl. Luminal perfusion or NaCl caused cell Cl to rise rapidly to values similar to those in Cl Ringer experiments. Perfusion of the tubule lumen with mixtures of NaCl and Na_2SO_4 Ringer, yielded a saturable dependence of cell Cl on luminal NaCl concentration identical to that seen in the Cl Ringer experiments. We conclude that the cell Cl activity is primarily determined by the NaCl concentration in the tubule lumen, that Cl entry across the luminal membrane is mediated and that Cl moves passively across the peritubular membrane.

Calculations of the transcellular chloride flux from microelectrode and tracer measurements give similar values, i.e. about 40% of the chloride normally reabsorbed by the tubules crosses the cells.

Proposed Course

Completion of the tracer flux study will allow calculations of all of the rate constants and permeabilities of the cell membranes and shunt pathway. Inhibitors of chloride transport will be evaluated by observations of their effects on cell chloride activity as well as the fluxes.

Publications

Spring, K. R. and G. Kimura: Chloride reabsorption by renal proximal tubules of Necturus. J. of Memb. Biol. 1977 (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01217-02 KE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Mechanism of epithelial fluid transport, the interspace volume clamp

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

PI: K.R. Spring, DMD, Ph.D., Sr., Staff Fellow, LKEM/NHLBI
Other: A. Hope, Ph.D., Visiting Fellow, LKEM/NHLBI

COOPERATING UNITS (if any)

Television Engineering Section, PSD, CC
10A/B1S33

LAB/BRANCH
Laboratory of Kidney & Electrolyte Metabolism

SECTION
Section on Electrolyte Transport

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland

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)

An optical-electrical system has been developed to enable precise, quantitative measurements of the dimensions of the intercellular spaces of living flat epithelia. Light absorption due to spaces between cells is measured under varying physiologic conditions. The shape of the spaces as well as the relationship between the volume of the spaces and physical forces across the tissue are being investigated.

Objectives

The primary goal of this investigation is the generation of information about the role of changes in the geometry of intercellular spaces in the regulation of epithelial fluid transport. The methods employed represent a unique blend of optical and electrical techniques developed specifically for this investigation. We use an on-line, real-time system for measuring the size and shape of the spaces.

In addition to the data about the geometrical effects of physical forces on epithelial properties information should be obtained on many of the physical properties of the tissue. Static pressure-volume curves of the cell membranes, as well as values for the hydraulic properties of the cell membranes should be obtainable.

Methods

The tissue of choice for these experiments in the gallbladder of the amphibian Necturus maculosus. The cells are 15 to 20 μM in diameter and 20 to 25 μM tall. The intercellular spaces are readily visualized and can be observed to open and close in response to hydrostatic or electric forces. This tissue is mounted in a specially designed chamber modified from a Dvorak-Stotler tissue culture chamber. The tissue is sandwiched between two mica plates so that approximately 0.2 cm^2 is exposed to both mucosal and serosal bathing solutions. Fluid constantly perfuses both mucosal and serosal baths. Electrodes enable potential measurements and passage of electric current. The total thickness of the chamber is 1.2 mm and it is so designed to fit within the optical path of a high power light microscope.

The chamber is placed on the stage of a Leitz inverted microscope equipped with brightfield optics. The condenser is replaced by an inverted objective lens to improve the focus and sharpness of the image. The objective lenses are standard high power dry and oil immersion. The viewing head is equipped with a beam splitter which is used to divert light from the image to a photomultiplier tube. The current output from the photomultiplier tube is used to indicate the size of the intercellular spaces. Adjustment of the slit of the photomultiplier enables measurements to be made from one intercellular space. The density of light absorption by the space is proportional to the dimensions of the space thereby providing a quantitative measure of change in morphology. Position and focus of the microscope is monitored by

observation of the preparation during measurements with the aid of a television camera. This camera is attached to the microscope beam splitter and contains an image intensifier so that low illumination levels may be achieved.

Major Findings

Gallbaldders mounted in our chamber are functioning normally and exhibit resistance, ion selectivity and fluid transport rates similar to published values. Photomultiplier current is directly proportional to the cross sectional area of the lateral intercellular spaces and varies as the focal plane is altered. Pressure-volume relationships have been established for the lateral spaces.

Proposed Course

We expect to obtain parameter values for the epithelium as a whole as well as for the lateral spaces. Electrical resistance of the spaces, fluid resistance of the cells and the spaces, and the pathways of fluid movement should all be measurable variables. The relationship between interspace geometry and various inhibitors of fluid or ion transport will be investigated.

Publications - None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL 01218-01 KE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Bicarbonate transport by proximal straight and cortical collecting tubules

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

PI: Thurman D. McKinney, M.D.
Other: Maurice B. Burg, M.D., Chief

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Kidney & Electrolyte Metabolism

SECTION
Electrolyte Transport

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1 2/3	1 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)

The purpose is to define the mechanisms of bicarbonate transport in individual isolated perfused nephron segments of rabbit kidneys. We are measuring bicarbonate transport in proximal straight and cortical collecting tubules and determining the dependence on sodium, chloride and carbonic anhydrase.

PHS-6040

Objectives:

Proximal tubules and cortical collecting tubules contribute significantly to renal bicarbonate absorption and urinary acidification. We showed previously that bicarbonate absorption by proximal tubules is dependent on sodium transport and is inhibited when sodium in the perfusate and bath is replaced by lithium or tetramethylammonium or when sodium transport is inhibited by removing potassium from or adding ouabain to the bath. There were no previous studies of bicarbonate transport by cortical collecting tubules. The purpose of the present studies was to measure bicarbonate transport in the collecting tubules and evaluate the effects of sodium and chloride and of carbonic anhydrase inhibition on this segment, as well as to complete the previous studies on proximal straight tubules.

Methods:

1. The method of isolation and perfusion of individual nephron segments from rabbit kidneys developed in this laboratory has been described in previous reports.

2. Total CO_2 content of nanoliter samples was measured by a microcalorimetric method, also previously described.

Major findings:

Proximal straight tubules

When sodium in the perfusate and bath was replaced by choline, bicarbonate absorption was inhibited, confirming the studies with other cation replacements.

The addition of the carbonic anhydrase inhibitor acetazolamide 10^{-4} M to the perfusate and bath caused complete inhibition of bicarbonate absorption. The inhibition of bicarbonate absorption by the drug in this segment was much more complete than previously described in micropuncture and clearance studies in other species.

We conclude that bicarbonate absorption in rabbit proximal straight tubules is dependent on carbonic anhydrase, as well as sodium.

Cortical collecting tubules

In tubules from normal animals there was on the average little net bicarbonate transport. Some tubules absorbed bicarbonate and some secreted it into the tubule lumen.

When animals were given NH_4Cl by gavage the day prior to the experiment their tubules in vitro absorbed bicarbonate. When they were pretreated with $NaHCO_3$ in this manner, their tubules in vitro secreted bicarbonate. Thus, pretreatment of animals with acidifying or alkalinizing salts induced a change in tubule epithelium which altered its bicarbonate transport in a direction favorable to maintenance of normal acid-base homeostasis of the intact animal. This mechanism most likely plays a role in excretion of acid and alkali loads. Bicarbonate secretion had not been described previously in mammalian renal tubules.

Ouabain 10^{-5} M did not affect either the secretion or the absorption of bicarbonate despite the fact that this concentration of the drug completly inhibits sodium transport by collecting tubules.

When the tubules were initially absorbing bicarbonate, replacement of sodium in the perfusate and bath by choline caused bicarbonate absorption to increase. In contrast, when tubules were initially secreting bicarbonate, the bicarbonate secretion was inhibited by replacement of sodium by choline.

On the other hand, bicarbonate absorption was inhibited by amiloride (10^{-5} M), another drug that also inhibits sodium absorption by these tubules. Conversely, amiloride enhanced bicarbonate secretion.

Replacement of sodium by choline, and addition of ouabain and amiloride inhibited the transepithelial potential difference regardless of whether the tubules were secreting or absorbing bicarbonate. The decrease in voltage presumably results from inhibition of sodium transport under these conditions.

Thus, bicarbonate secretion but not absorption appears to depend on sodium. Neither bicarbonate secretion or absorption correlated with the transepithelial potential difference. The fact that bicarbonate absorption in collecting tubules does not depend on sodium contrasts markedly with the dependence of this process on sodium in proximal tubules.

Bicarbonate secretion was unaffected when chloride in the perfusate and bath was replaced by nitrate or methylsulfate. Therefore, bicarbonate secretion does not require the presence of chloride in cortical collecting tubules, as it does in turtle urinary bladders.

Acetazolamide 10^{-4} completely inhibited both bicarbonate secretion and absorption. Thus, both processes require carbonic anhydrase activity.

Significance

Acidification and alkalinization of the urine are essential for homeostasis of body pH. The studies on isolated renal tubules are greatly expanding our knowledge of the physiology and pathophysiology of this system.

Proposed Course

1. Three new microanalytic techniques are being developed to expand the scope of the studies: a) glass microelectrode to measure the pH of nanoliter samples of tubule fluid. b) Combination of (a) with an antimony electrode that generates hydroxyl or hydrogen ions to measure titratable acidity. c) a technique for measurement of $H^{14}CO_3$ flux.

2. With the combined use of the new and old techniques we will attempt to answer the following questions: Do the nephron segments other than proximal tubules and collecting tubules acidify or alkalinize the tubule fluid? Do any other segments besides the cortical collecting tubules adapt their response measured in vitro to acidosis or alkalosis in the living animals? Is induction of specific enzymes the cause of adaptations, and, if so, which enzymes? Do acidification and alkinization depend on exogenous bicarbonate? Which molecular species are transported: bicarbonate, hydrogen ions, or others? What are the bicarbonate and CO_2 permeabilities of the various nephron segments?

Publications

Burg, M., Patlak, C., Green, N. and Villey, D.: Organic solutes in fluid absorption by renal proximal convoluted tubules. Am. J. Physiol. 231:627-637, 1976.

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Burg, M. and Green, N.: Role of monovalent ions in the reabsorption of fluid isolated perfused proximal renal tubules of rabbit. Kidney. Internat. 10:221-228, 1976.

Warnock, D., Burg, M. and Orloff, J.: Urinary acidification: CO_2 transport by the rabbit proximal straight tubule. Am. J. Physiol. 1:F20-25, 1977.

Burg, M. B.: Studies on sodium transport in isolated proximal tubules. Macy Conference, Charleston, S. C. (in press)

Burg, M. and McKinney, T. D.: Bicarbonate secretion by rabbit cortical collecting tubules in vitro, J. Clin. Invest. (in process)

McKinney, T. D. and Burg, M. B.: Bicarbonate absorption by rabbit cortical collecting tubules in vitro. Am. J. Physiol. (in press)

McKinney, T. D. and Burg, M. B.: Bicarbonate transport by rabbit cortical collecting tubules: effect of acid & alkali loads in vivo on transport in vitro. J. Clin. Invest. (in press)

McKinney, T. D. and Burg, M. B.: Bicarbonate and fluid absorption by renal proximal straight tubules. Am. J. of Physiol. (in press)

Burg, M. B. and Green, N.: Bicarbonate transport by isolated perfused rabbit proximal convoluted tubules. Am. J. Physiol. (in press)

Burg, M. B., Warnock, D. G. and Patlak, C. S.: Contribution of leaked load to solute transport by renal tubules. Am. J. Physiol. (in process).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HL 01219-01 KE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Tissue culture of epithelial cells from the urinary tract

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

PI: J. S. Handler, M.D., Hd., Sec. on Membrane Metabolism LKEM/NHLBI

Other: M.B. Burg, M.D., Chief, LKEM/NHBLI
M. K. Sahib, Ph.D., Visiting Scientist, Sec. on Membrane Metabolism/LKEM/NHLBI
A.S. Preston, Chemist, Sec. on Membrane Metabolism/ LKEM/ NHLBI
Nordica Green, Chemist, Sec. on Electrolyte Transport/ NHLBI
R. Steele, Ph.D., Physical Scientist, TD/NHLBI

COOPERATING UNITS (if any)
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LAB/BRANCH
Laboratory of Kidney & Electrolyte Metabolism

SECTION
Section on Membrane Metabolism

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.9	0.1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

This program was initiated to obtain in quantity homogenous populations of cells that possess transport properties of interest to the nephrologist. Cell lines currently in continuous culture will be surveyed for transport properties and responses to hormones and drugs similar to those of renal tubules. In addition to the established cell lines, we will attempt to start primary culture of cells from different segments of the rabbit nephron and from the toad urinary bladder.

Project Description

Objectives

Renal physiology has progressed to the point where significant advancement in understanding transport and its control can be expected to come from study of these processes on a subcellular level. Current efforts in this area involve the study of vesicles prepared from apical and from basolateral plasma membranes. The vesicles are assumed to be composed primarily of membranes from the most common cell type in the region of the kidney used for the preparation. Preparations from the cortex are assumed to be composed primarily of membranes from the proximal convoluted tubule, and those from the medulla are assumed to be composed primarily of membranes from the collecting duct.

This approach is useful but has two major deficiencies; 1) The preparations are impure in that the starting material is a heterogenous population of cells, and 2) the segments of the nephron which form only a small fraction of the renal mass cannot be investigated in this fashion. The only method currently at hand for obtaining a pure population of epithelial cells from different nephron segments is by micro-dissection (developed largely in LKEM), a technique that yields extremely small amounts of tissue. Cell culture offers the possibility of obtaining large amounts of a particular type of renal epithelial cell. It is the purpose of this program to develop techniques for the production of large amounts of homogenous renal epithelial cells and to study their function on a cellular and subcellular level.

Methods employed and major findings

Our hope for success in this project rests in large part on the previous demonstration by others that cultured epithelial cells form sheets, which orient with the urinary surface of the cells away from the plastic support and which transport vectorially. We have obtained several continuous lines of renal epithelial cells and have found evidence for vectorial transport (dome formation) in two lines, one of canine origin and one of bovine origin. The canine cells possess adenylate cyclase that is stimulated by vasopressin and by glucagon (M. Saier, personal communication), and the bovine cells possess adenylate cyclase stimulated by PGE_1 and isoproterenol. Other continuous cell lines of rat and of rabbit origin have been examined and put aside because they do not form domes and do not possess adenylate cyclase that is hormone sensitive.

Another potential source of epithelial cell is primary culture of proximal convoluted tubules dissected from rabbit kidneys. We have grown the epithelial cells (as well as adherent fibroblasts) in hormone supplemented enriched medium (Coon's modification of Ham's F-12). In addition, we have grown epithelial cells from the toad urinary bladder in Coon's modification of Ham's F-12 medium supplemental with crude beef embryo extract, toad serum, dexamethasone, and cyclic AMP. The toad bladder cells have been carried in culture for two months to the present and have continued to form domes during this time.

Significance to Biomedical Research and Program of the Institute

The availability of large amounts of homogenous, transporting renal epithelial cells will be a major investigative tool for further study of renal transport and its control by hormones and drugs.

Proposed Course

Other continuous lines of renal epithelial cells will be tested for vectorial transport and responses to hormones. The different segments of the nephron will be dissected and used to start primary cultures. The culture media for the primary cultures of epithelial cells will be supplemented with hormones, nutrients, and other factors in an attempt to obtain cell growth that is sufficient for cloning. Cloning should yield a homogeneous population of cells which can be used for detailed study of transport and biochemical properties.

Publications - None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01220-01 KE

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

The effect of adrenal steroid hormones on prostaglandin metabolism in toad urinary bladder

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

PI: Randall Zusman, M.D., Clinical Associate, Hypertension-Endocrine Branch/NHLBI

Other: Harry Keiser, M.D., Deputy Chief, Hypertension-Endocrine Branch/NHLBI

Joseph S. Handler, M.D., Hd. Section on Membrane Metabolism/LKEM/NHLBI

COOPERATING UNITS (if any)

Hypertension-Endocrine Branch/NHLBI

LAB/BRANCH

Laboratory of Kidney & Electrolyte Metabolism

SECTION

Section on Membrane Metabolism

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20014

TOTAL MANYEARS: 0.5	PROFESSIONAL: 0.5	OTHER:

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Adrenal steroid hormones inhibit prostaglandin synthesis by the toad urinary bladder in vitro. This effect explains, in part, the enhancement by adrenal steroids of the water permeability response to vasopressin. Studies with analogs of adrenal steroid hormones are compatible with the interpretation that this is a glucocorticoid effect.

PHS-6040

Project Description

Previous studies in this laboratory have shown that toad urinary bladders incubated with adrenal steroid hormones have a greater water permeability response to vasopressin, to cyclic AMP, and to theophylline than bladders depleted of the effects of adrenal steroids. Measurement of cell cyclic AMP, the intracellular mediator of the action of vasopressin, demonstrated markedly higher cyclic AMP levels in response to vasopressin in steroid treated bladders compared to steroid depleted bladders. These results would be explained if adrenal steroids inhibited cyclic nucleotide phosphodiesterase which is the enzyme that destroys cyclic AMP. Assay of cyclic nucleotide phosphodiesterase activity in homogenates of epithelial cells from adrenal steroid treated tissue revealed only 20 percent inhibition which might not explain the entire effect on cell cyclic AMP levels. Therefore, we have investigated the possibility that adrenal steroids exert a second effect on cyclic AMP metabolism in the bladder.

It is known from previous studies in this laboratory that exogenous prostaglandin E (PGE) reduces cyclic AMP accumulation by inhibiting adenylate cyclase activity in toad bladder epithelial cells. This year (see Annual Report ZO1 HL 01908-02 HE) we found that vasopressin stimulates PGE production by the bladder. In the present study we examine the effect of adrenal steroid hormones on PGE biosynthesis in the toad urinary bladder, since reduced synthesis of PGE in bladders incubated with adrenal steroid hormones could be a second effect enhancing the cyclic AMP mediated water permeability response to vasopressin.

Major Findings

1. Incubation with adrenal steroid hormones results in marked depression of the rate of PGE biosynthesis. The inhibitory effect of adrenal steroids is evident on basal as well as vasopressin stimulated PGE biosynthesis.

2. The inhibition by adrenal steroids of cyclic nucleotide phosphodiesterase activity also contributes significantly to the increased water flow response. Bladders incubated with adrenal steroid hormone had a greater water flow response to vasopressin than did steroid depleted bladders even when the effects of endogenous PGE production were eliminated by addition of naproxen, a potent prostaglandin synthetase inhibitor.

3. The step in PGE biosynthesis that is inhibited in adrenal steroid treated tissue is acylhydrolase (phospholipase). This step causes release from storage pools of free arachidonic acid, the precursor of PGE.

ND →

4. Comparison of the relative effectiveness of analogs of adrenal steroid hormones indicates that the effect on PGE biosynthesis and the related enhancement of the water permeability response is probably a glucocorticoid effect.

Significance to Biomedical Research and Program of the Institute

The results of this study are of importance in understanding the factors regulating the response of urinary epithelial cells to vasopressin. The observations also clarify the role of PGE in these tissues. Prostaglandins, which affect the response to vasopressin are in turn regulated by other hormones. Thus, glucocorticoids affect prostaglandin synthesis and the prostaglandins modulate the effect of vasopressin. This newly described regulatory system may also exist in other tissues.

Proposed Course - Project is completed.

Publications - None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01221-01 KE

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Vasopressin evoked refractoriness of water permeability response of toad bladder

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

PI: Joseph S. Handler, M.D., Head, Sec. on Membrane Metabolism/LKEM
Other: Agnes S. Preston, Chemist, LKEM

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Kidney & Electrolyte Metabolism

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Section on Membrane Metabolism

INSTITUTE AND LOCATION

NHLBI, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

It has been observed that incubation of the toad bladder with high concentrations of vasopressin results in a reduced water permeability response to subsequent stimulation by vasopressin. This study is intended to elucidate the mechanism of vasopressin elicited refractoriness to vasopressin.

Project Description:

Objectives - Hormone elicited refractoriness to stimulation by the hormone has been described recently in a number of tissues. In the course of other studies we have observed that incubation of the toad urinary bladder in vitro with high concentrations of vasopressin (AVP) results in reduced responsiveness of the water permeability response upon subsequent exposure to vasopressin. This study is intended to identify the mechanism of the vasopressin elicited refractoriness.

Major Findings:

The refractoriness of the water permeability response is related to the duration of the initial incubation with vasopressin and the concentration of vasopressin used. 10 mU/ml of vasopressin for 60 min or 50 mU/ml for 15 min elicit refractoriness, but 10 mU/ml for 15 min or 2 mU/ml for 60 min do not. The time required for recovery from refractoriness elicited by AVP is also related to the conditions of the initial exposure. Bladders manifest refractoriness 18 hours after incubation with 50 mU/ml AVP for 120 min, but appear to be fully recovered after 18 hours if the initial exposure to 50 mU/ml AVP was for only 15 min. The development of refractoriness and recovery to normal sensitivity do not appear to depend upon protein synthesis in that refractoriness and recovery are not altered by the addition of actinomycin -D or cycloheximide, in concentrations known to inhibit protein synthesis in the bladder. In some other tissues it has been found that hormone elicited refractoriness to stimulation by the hormone is the result of loss of receptors for the hormone. One manifestation of this is less hormonal stimulation of adenylate cyclase activity. Reduced hormonal stimulation of adenylate cyclase activity in toad bladder epithelial cells was found after 18 hours of incubation with vasopressin but was not detected after two hours of incubation with 50 mU/ml of vasopressin, when there is considerable refractoriness of the water permeability response to the hormone. It is possible that methods do not permit detection of small loss of sensitivity to hormonal stimulation. It seems more likely, however, that refractoriness occurs by more than one mechanism during the shorter as well as the 18 hours of incubation with vasopressin. Bladders incubated previously with vasopressin are also refractory to stimulation of water permeability by exogenous cyclic AMP. We have been unable to rule out the possibility that refractoriness to stimulation by cyclic AMP is the result of failure of exogenous cyclic AMP to enter the epithelial cells. We have also been unable to detect a change in cyclic AMP dependent protein kinase in the soluble

fraction of the epithelial cells from refractory bladders. This enzyme has been implicated in the cyclic AMP mediated response to vasopressin.

Significance:

This study has defined a situation in vitro in which the response of urinary epithelia to hormonal stimulation is altered. There is evidence that similar vasopressin elicited refractoriness to stimulation by vasopressin exists in mammalian kindey in vitro.

Proposed Course:

Bladders will be made refractory by incubation with vasopressin and then cyclic AMP levels will be measured following stimulation by vasopressin. This experiment should reveal whether there is reduction in vasopressin elicited accumulation of cyclic AMP in refractory tissue

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01222-01 KE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Mechanism of transport of calcium and other ions by the thick ascending limb of Henle's loop.

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

PI: James E. Bourdeau, M.D., Ph.D.
Other: Maurice B. Burg, M.D.
Nordica Green

COOPERATING UNITS (if any)
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LAB/BRANCH
Laboratory of Kidney & Electrolyte Metabolism

SECTION
Section on Renal Mechanisms

INSTITUTE AND LOCATION
NHLBI, NIH Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1 2/3	1 1/3	1/3

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Calcium (and other ion) transport in the thick ascending limb of Henle's loop is being studied in isolated perfused segments of rabbit renal tubules in vitro. Isotopic flux studies and electron probe microanalysis of tubular fluid reveal that significant amounts of calcium are transported and that a large component of this transport may be passive secondary to transepithelial voltage.

Objectives

We previously found that this tubule segment actively absorbs chloride. The active chloride transport caused the voltage to be positive in the tubule lumen, and the positive voltage, in turn, caused absorption of sodium and potassium. The absorption of the cations was largely passive. Diuretic drugs inhibited the active chloride transport and caused the voltage to decrease. As a result the absorption of sodium and potassium decreased, explaining why the diuretics cause increased urinary excretion of sodium and potassium. The purpose of the present studies is to test whether the voltage also causes passive absorption of other ions. Calcium is of special interest in this regard because a substantial fraction of the absorption of calcium occurs in the thick ascending limb, and calcium, being a divalent cation, should be strongly affected by electrical forces.

Methods Employed

The technique that we previously developed to perfuse single dissected nephron fragments in vitro provides a means to study directly the function of each tubule segment. Using this technique only nanoliter quantities tubule fluid are available for analysis, however, which has limited its application. Electron probe microanalysis provides a means for elemental analysis of picoliter samples. Therefore, we have adopted this technique for our studies. Picoliter volumes of tubule fluid are pipetted onto beryllium blocks, and the water is evaporated leaving small deposits of dried solute. Since there is no electron probe at NIH, the blocks are flown to Boston where Dr. Lechene analyzes the sample deposits with his electron probe. The probe directs a uniform electron beam on the deposits. X-rays, which have characteristic wave lengths for each element, are produced. The x-rays are analyzed to identify and measure the quantity of the elements present. After nearly one year of effort we have developed this demanding and tedius technique to the point where we can make measurements with sufficient accuracy to analyze tubule fluids. We are now analyzing sodium, potassium, chlorine, calcium, phosphorus, and magnesium. In addition, we have worked out a method for measuring ^{45}Ca fluxes.

Major Findings

1. ^{45}Ca flux was studied under two conditions which correspond to the beginning of the thick ascending limb, where the voltage is relatively low, and the end, where the voltage

END

is relatively high. The difference in voltage results from the decrease in NaCl concentration in the tubule lumen as salt is absorbed and a NaCl dilution potential develops. With equal sodium concentration in the perfusate and bath (as at the beginning of the thick ascending limb) the mean voltage was +6mv and the ^{45}Ca flux from lumen to bath was 0.17 pEq cm^{-1} s^{-1}. With a low sodium concentration in the lumen with respect to the bath (as at the end of the thick ascending limb) the mean voltage was +21 mV, and the ^{45}Ca flux from lumen to bath was 0.35 pEq cm^{-1} sec^{-1}. This finding provides important preliminary evidence that calcium is passively absorbed in this segment as are sodium and potassium.

2. Electron probe analysis of tubule fluid under conditions similar to those at the beginning of the thick ascending limb confirmed the previous findings with respect to sodium, chloride, and potassium. There was also net absorption of calcium under conditions similar to those at the end of the thick ascending limb.

Significance

Renal handling of calcium is important for its homeostasis. The thick ascending limb is an important site of calcium absorption. Elucidation of the transport mechanism for calcium (and the other elements) in this segment will provide important insights into the physiology and pathophysiology of their renal handling.

Proposed Course

1. We shall investigate the net flux of calcium (and the other elements) using the electron probe to determine the dependence of the fluxes on voltage and on the action of hormones and drugs.

2. We shall complete the ^{45}Ca studies by measuring the flux from bath to lumen under the conditions already studied.

3. We shall attempt to "clamp" the voltage at extreme values (positive and negative in the lumen) by the use of salts other than NaCl to create dilution and bionic potentials and determine the effect on fluxes measured with the electron probe and ^{45}Ca.

4. The new analytical techniques will be applied to other nephron segments in order to determine their mechanisms of calcium, phosphate, and magnesium transport and the means by which the transport is controlled.

Publications

Burg, M. and Stoner, L.: Renal tubular chloride transport. Am. Rev. of Physiology. 38:37-45, 1976.

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Stoner, L. C.: Isolated, perfused amphibian renal tubules: The diluting segment. Am. J. Physiol. (in press)

Burg, M. B. and Stephenson, J. L.: Transport characteristics of the loop of Henle in Physiological Basis for Disorders of Biomembranes, Hoffman and Fannestil eds. (in process)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01223-01 KE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

A second regulatory effect of norepinephrine in toad urinary bladder

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

PI: David ShermanM.D., Guest Investigator, LKEM/NHLBI
Other: Agnes S. Preston, Chemist, LKEM/NHLBI
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COOPERATING UNITS (if any)

None

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Laboratory of Kidney & Electrolyte Metabolism

SECTION
Section on Membrane Metabolism

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.5	0.1

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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 earlier studies we showed that norepinephrine, acting as an alpha adrenergic agonist, inhibited the water permeability response of the toad urinary bladder to vasopressin and to theophylline, but not that to cyclic AMP, because norepinephrine inhibited adenylate cyclase activity. In this study we are examining a second and possibly related effect of norepinephrine, increased responsiveness to theophylline after withdrawal of norepinephrine from the incubation solution. Experiments will be performed to identify the type (alpha vs. beta) of adrenergic activity involved, the site of the effect (adenylate cyclase, cyclic nucleotide phosphodiesterase, etc.).

PHS-6040
(Rev. 10-76)

Project Description

Objective

Previous studies from this laboratory demonstrated that norepinephrine inhibits the water permeability response of the toad urinary bladder to vasopressin and to theophylline but not that to cyclic AMP. This effect has been shown to be the result of inhibition of in situ adenylate cyclase activity by the alpha adrenergic agonist. Recently, in the course of other studies we have observed a second effect of incubation with norepinephrine. Following withdrawal of norepinephrine from the solution in which the bladder is incubated there is an increase in the water permeability response to vasopressin, to cyclic AMP, and to theophylline. It is the purpose of this project to study this second effect of norepinephrine.

Major Findings

1. The second, stimulatory effect of norepinephrine is evident within 15 min. and is greater following more prolonged incubation with norepinephrine.

2. Norepinephrine is effective in concentrations as low as 3×10^{-6} M.

3. The effect of norepinephrine can be blocked by adding the alpha adrenergic blocking agent phentolamine during the initial incubation with norepinephrine, a result we interpret as indicating that the second effect of norepinephrine is an alpha adrenergic effect. Phentolamine has no effect when added after the norepinephrine is withdrawn (during the period of the increased water permeability response). Propranolol, a beta adrenergic blocking agent is without effect on this second action.

Significance to Biomedical Research and the Program of the Institute

The second effect of norepinephrine was not previously described but there may be an analogous response in certain lines of neuroblastoma cells that have been studied in tissue culture in the Laboratory of Biochemical Genetics, NHLBI. Adenylate cyclase activity increases in these cells following prolonged incubation with opiates, carbachol, or norepinephrine.

Proposed Course

Conditions optional for demonstration the second effect of norepinephrine will be identified and used in studies designed to identify the biochemical site of action. We will assay adenylate cyclase activity, cyclic nucleotide phosphodiesterase activity, and epithelial cell cyclic AMP content. A similar protocol will be used to see whether opiates or carbachol produce this effect as they do in neuroblastoma cells. Depending on the results of the foregoing experiments, we may examine whether incubation with norepinephrine subsequently affects sodium transport, a second important transport process that is stimulated by cyclic AMP in the toad urinary bladder.

Publications - None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HL 01224-01 KE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Control of sodium and potassium transport by isolated rabbit tubules

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

PI: George J. Schwartz, M.D., Guest Worker, LKEM/NHLBI
Other: Maurice B. Burg, M.D., Chief, LKEM/NHLBI/NHLBI

COOPERATING UNITS (if any)
None

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Laboratory of Kidney & Electrolyte Metabolism

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Section on Electrolyte Transport

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NHLBI, NIH, Bethesda, Md. 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Sodium and potassium balance was altered in rabbits by drugs or by dietary changes. Then, the effect, if any, on sodium and potassium transport and the transepithelial voltage across their isolated perfused cortical collecting tubules in vitro was determined.

PHS-6040
(Rev. 10-76)

Objectives

It is known from previous studies that alterations in the amounts of sodium and potassium in the diet given to rabbits, causes differences in the transepithelial voltage across their cortical collecting tubules when the latter are removed and studied in vitro. (Frindt & Burg, Kidney Internat. 1:224, 1972) Also, administration of desoxycortisone acetate to the rabbits alters sodium, potassium, and chloride transport by the isolated collecting tubules, as well as the voltage (O. Neil, R. Ph.D. Thesis, University of Illinois, 1976). Apparently, the dietary and pharmacological manipulations induced changes in the tubules which persisted for several hours in vitro. Presumably, the changes also were effective in vivo and contributed to the control of urinary excretion of salt and potassium by the living rabbits. In the present studies the in vitro function of cortical collecting tubules was measured after rabbits had been subjected to systematic changes in diet or given drugs. The purpose was to evaluate to what extent long-lived changes of tubule function are induced which might contribute to the control of urinary excretion.

Methods

The rabbits given special diets and/or drugs were killed and their urine and blood examined for changes in electrolyte composition. Then, their cortical collecting tubules were perfused in vitro, using the method previously described, in order to measure electrolyte transport and transepithelial voltage.

Major Findings

In preliminary studies with rabbits on the usual NIH diet we found that tubule viability and transport were unaffected by the presence or absence of protein in the bath, or by the presence or absence of organic solutes (lactate, glucose, alanine, citrate) in the perfusate. Removal of bicarbonate from the perfusate caused a 50% increase in transepithelial voltage, but no significant change in sodium and potassium transport. In subsequent experiments we used an artificial solution for the perfusate and bath which contained organic solutes and 25 mM bicarbonate, but no protein.

High doses of hydrochlorothiazide added to bath and lumen caused a 60% increase (more negative) in transepithelial voltage, and a 20% decrease in sodium absorption. Clearance studies using this drug in rabbits were performed for us by Dr. G. Fanelli of Merck, Sharp & Dohme. He found that the rabbit, unlike rat, dog, cat and man, responds poorly to hydrochlorothiazide. Therefore, we deferred further studies of the effect of hydrochlorothiazide on the renal tubules.

The following controlresults were obtained with collecting tubules from rabbits maintained on the regular NIH diet: transepithelial voltage -17 mV, sodium flux from lumen to bath 7 peq and from bath to lumen 1.5 peq/cm/sec and net potassium secretion 2 peq/cm/sec. These values decreased gradually by approximately 20% over 2 hours of study.

Chronic salt loading with 0.9% NaCl drinking water did not affect the sodium or potassium transport, even though urinary sodium/potassium ratios markedly increased. Addition of aldosterone to these tubules in vitro did not affect transport or voltage during 30-90 minutes. These animals were excreting the additional sodium (high urinary Na/K ratio), but the control of excretion evidently was not by long-lived changes in the collecting tubules.

Administration of NH_4Cl resulted in 100% increase in transepithelial voltage and potassium secretion without a significant change in sodium transport.

Acute furosemide-induced volume contraction (10% of body weight) associated with a fall in plasma Na to 128 meq/l failed to affect sodium or potassium transport or the voltages. These animals had very little salt in their urine, but the control of salt excretion apparently did not involve long term changes in the collecting tubules.

Low potassium, high sodium diet for one week resulted in severe hypokalemia associated with failure to grow, hair loss and ileus. Sodium absorption by the collecting tubules decreased 50% and potassium secretion decreased 75% in this group of rabbits. Addition of aldosterone in vitro failed to affect transport or voltage even though plasma aldosterone was undetectable at the time of study. The decreased potassium secretion by the collecting tubules presumably is part of the homeostatic response to potassium deprivation in these animals, but there is no evidence that it is due to decreased aldosterone. The severity of the changes in the animals, however, raises the question of whether hypokalemic nephropathy may have been present and contributed to the result.

Low salt diet for 1-3 weeks caused Na absorption to increase by 50% and the voltage by 150%. Urinary Na/K ratios decreased markedly while plasma aldosterone levels increased 6-fold, compared with controls. The change in Na transport by the collecting tubules is in the right direction for the homeostatic control of its excretion in the whole animal and the change may be due to the increased level of aldosterone.

Desoxycorticosteroid acetate administration caused large increases in sodium absorption (200%), potassium secretion (400%) and voltage (300%). Urinary Na/K ratio was equal to that of untreated rabbits on the same diet, suggesting that the animals were in a steady state. Assuming that the collecting tubules were also transporting at enhanced rates in the intact animals, there must have been compensating changes in the other tubule segments to maintain balance. The response of the tubules exceeded that to low salt diet, which raises the question whether the drug had pharmacologic effects in addition to its mineralocorticoid action.

Significance

The factors controlling salt and potassium excretion are poorly understood. The long lived changes in the collecting tubule identified in these studies may be important contributing factors. If so, their elucidation should greatly increase the knowledge of this area of renal physiology and pathophysiology.

Proposed Course

1. In order to test whether more extreme salt deprivation might boost the level of endogenous mineralocorticoids to the point where the transport of the collecting tubules matches that given desoxycorticosterone acetate, a low sodium diet will be provided for 10 days, during which time furosemide 1 mg/kg/IV will be given thrice.

2. A moderately low potassium diet in the absence of Na loading will be employed to ascertain whether less severe hypokalemia, causes transport to decrease.

3. Rabbits will be given dexamethasone in order to assess the effect of a pure glucocorticoid agent on transport and voltage in collecting tubule. This study is necessary because large doses of desoxycorticosterone acetate are known to cause glucocorticoid effects.

4. Finally, other nephron segments will be studied under the same conditions in order to test whether they also change in function.

Publications

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01225-01 KE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Control of cyclic nucleotide phosphodiesterase

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

PI: Gordon J. Strewler, Sr. Scientist/LKEM/NHLBI
Other: Jack Orloff, Scientific Director, NHLBI
V. C. Manganiello, Sr. Staff Fellow, LCM/NHLBI
M. Vaughan, Chief, LCM/NHLBI

COOPERATING UNITS (if any)
None

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Laboratory of Kidney & Electrolyte Metabolism

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INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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1	1	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

We have studied factors controlling cyclic nucleotide phosphodiesterase in rat renal cortex. The cyclic GMP phosphodiesterase activity, but not the cyclic AMPphosphodiesterase activity, is decreased in rats treated with dexamethasone. In addition an endogenous activator of phosphodiesterase, located in a particulate fraction, is released by osmotic shock and can activate a cAMP phosphodiesterase and a cGMP phosphodiesterase. The activator differs from a previously described protein activator of phosphodiesterase in that it is heat labile and does not require calcium for activity. It can also activate phosphodiesterase from other sources, including rat liver. On sucrose density gradient centrifugation the activated form of activator-sensitive enzymes has a lower sedimentation velocity than does the unactivated form, suggesting that the activated form has a lower molecular weight. This is consistent with the interpretation that the activator is a protease.

Objectives

It is established that cyclic nucleotide phosphodiesterase (E.C. 3.1.4.17) in several tissues is under hormonal control. Glucocorticoids, prostaglandins and insulin are among the hormones known to affect phosphodiesterase. The mechanisms whereby hormones control activity of this enzyme are poorly understood. Phosphodiesterase is also sensitive to a heat stable, calcium dependent protein activator and to the proteolytic enzymes trypsin and chymotrypsin; but whether agents such as these might mediate hormone effects is unknown. The initial objective of the subsequent effort has been directed at characterizing a new endogenous activator of phosphodiesterase, which we have identified in rat kidney cortex.

Methods

Phosphodiesterase activity is measured by a previously described method (J. Biol. Chem. 252:1448-1452, 1977). In experiments to test the effect of glucocorticoids, dexamethasone (1 mg/Kg) or vehicle is injected daily for three days; rats are sacrificed on the fourth day and phosphodiesterase activity measured in homogenates of renal cortex. Kidney phosphodiesterase activator is solubilized by hypotonic lysis of a particulate fraction (see below) and partially purified by ammonium sulfate precipitation. Phosphodiesterases from rat kidney or rat liver have been partially purified by chromatography on DEAE cellulose. Certain of them are used as substrates for the activator.

Major Findings

1. In rats treated with dexamethasone cyclic GM phosphodiesterase activity is decreased. Cyclic AMP phosphodiesterase activity is unchanged. These are similar to glucocorticoid effects on phosphodiesterase in certain other tissues (J. Clin. Invest. 51: 2763-2767, 1973, J. Biol. Chem. 252:1448-1452, 1977_.

2. Cyclic AMP phosphodiesterase activity increases with time in homogenates of rat renal cortex; the increase is more rapid in hypotonic medium than in one of physiologic tonicity. We have identified a phosphodiesterase activator in this tissue. In isotonic media the activator is associated with a particulate fraction from which it can be released by osmotic shock, resulting in rapid activation of soluble cyclic AMP phosphodiesterase. Kidney phosphodiesterase activator is different from a previously described protein activator of phosphodiesterase (J. Biol. Chem. 246:2859-2869, 1971) in that it is heat labile and its effect does not require calcium.

3. Phosphodiesterases of rat kidney cortex and rat liver have been partially resolved by chromatography on DEAE cellulose, and cyclic GMP and cyclic AMP phosphodiesterases sensitive to activator have been identified in both tissues. The activated form of these enzymes has a lower sedimentation velocity than does the unactivated form, which suggests (but does not prove) that kidney phosphodiesterase activator decreases the molecular weight of enzymes sensitive to it. This interpretation is consistent with the view that the activator is a proteolytic enzyme.

4. Several different phosphodiesterases were found in rat kidney cortex. In addition to a high affinity cyclic AMP phosphodiesterase and a high affinity cyclic GMP phosphodiesterase which had previously been described in this tissue, we have identified a phosphodiesterase whose hydrolysis of cyclic AMP is markedly enhanced in the presence of cyclic GMP. This may be analogous to a rat liver phosphodiesterase for which cyclic GMP is an allosteric effector of cyclic AMP hydrolysis.

Significance to Biomedical Research

1. The observation that in hypotonic media an activator is released from the particulate fraction, activating phosphodiesterase, is relevant to those attempting to purify native phosphodiesterase. Indeed this finding may explain the interesting observation that a new form of phosphodiesterase appears with aging of rat liver extracts (J. Biol. Chem. 248:1334-1340), 1973).

2. It is possible that phosphodiesterase activators similar to the one we have characterized may mediate the effects of certain hormones and other agents on the enzyme. If so, the activators may be of importance under some circumstances in regulating cyclic AMP content.

Proposed Course

1. Attempts currently under way to purify and further characterize the activator from rat kidney will be continued.

2. Studies are being undertaken to determine whether hormones and the other agents known to affect phosphodiesterase activity change the activity of this activator and similar ones in other tissues.

Publications - None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01226-01 KE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Molecular mechanisms of transport in human red blood cell membranes

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

PI: Z. Ioav Cabantchik, Ph.D., LKEM/NHLBI
Other: Moshe Barzilay, Ph.D., LKEM/NHLBI
Doris Jones, Chemist, LKEM/NHLBI

COOPERATING UNITS (if any)
None

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NIH, NHLBI, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2	.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The goal of this project is to elucidate the mechanism by which human red blood cell membranes facilitate the diffusion of two distinctly different types of molecules: anions and nucleosides. The main line of work emphasizes the identification, isolation, chemical characterization and reconstitution of the critical membrane components of the two transport systems. The ultimate goal is to provide functional isolated systems in which transport processes can be dissected out into molecular events with the aid of a battery of physioco chemical techniques which include fluorescence, ESR and NMR spectroscopy.

1. Methodologies

Identification and Isolation of transport systems

The obvious approach for identifying functional components of transport in biological membranes is to affix markers highly selective for the particular systems. This should serve as a basis for tracking the transport system and expedite the development of fractionation procedures needed for isolating the critical components. The rational which we adopted for designing markers is based on the systematic alteration of the substrate structure that leads to high affinity probes, i.e. competitive inhibitors which display 3 to 4 orders of magnitude higher affinities than the natural substrate. These properties are combined with covalent binding capacity to provide the definitive surface anchoring so as to attain a firm tagging confined largely to the microenvironment of the inhibitory site. The highly specific affinity probes capable of either reversible or irreversible binding are also an asset for reconstructing the chemical architecture of substrate binding sites.

2. Major Findings

a. Anion transport system

The anion transport system of human red blood cells has been extensively characterized by us and others both in terms of its kinetic as well as chemical properties. The role played by various chemical probes has undoubtedly been the key for recent developments in the field. We have recently introduced two new methods to isolate the putative functional components of transport in a high degree of purity. In the first, they are isolated together with the native lipids of the membrane and in the second they are reconstituted in vesicles of known phospholipid composition. The vesicles rich in 100,000 dalton polypeptides (band 3 in SDS-acrylamide gels) show anion transport properties commensurate with those of the intact cells. These include anion specificity, susceptibility to specific inhibitors, modes of permeation (exchange/net), energy of activation (Ea), and specific anion exchange capacity. In the reconstituted system the Ea for sulfate exchange is approximately 1/2 the value found in intact cells, (17 as compared to 30 kcal/mole). The difference stems primarily from the ostensible lack of cholesterol and sphingomyelin in the system reconstituted with pure lecithins. Another major difference in vesicles is the insensitivity of sulfate self exchange for H^+ concentration below pH 6.3. This property is tentatively attributed to the lack of a functionally unrelated but structurally important protein present in intact cells, i.e., spectrin.

b. Nucleoside transport

The nucleoside transport system of human red blood cells is recognized as an important component of the erythrocyte membrane. An exhaustive description of its kinetic properties has recently been completed and published by us. This system was chosen for further molecular studies on the basis that its kinetic properties are consistent with those of a simple carrier or gate mechanism and provides therefore an ideal experimental object for studies of structure-function relationships.

Identification of functional components

We proceeded in our work by designing high affinity photoreactive probes specific for nucleoside transport entities. Among many synthesized and tested, APMI, p-azidophenacyl-S-6 mercapto purine riboside has been selected, and its inhibitory effects studied in detail. It has been found that in the dark it acts as a reversible competitive inhibitor of uridine self exchange (Ki=0.5 μMolar as compared to Ks= 1.3 μMolar at 25°C, pH 7.4). Upon photoactivation to produce reactivity nitrenes from the unreactive azido groups, the reversibly bound probes are affixed on the membrane and an irreversible inhibition is produced. From classical Johnson and Eyring plots we establish a 1:1 correlation (i.e. stoichiometry) between binding sites and inhibition. An estimate of 10,000-15,000 carriers per cells has been obtained.

3. Proposed Course

a. Anion Transport

Present work with band 3 isolated vesicles is aimed at evaluating the factors which contribute to the pH response of anion exchange. In the acidic range, it is assumed that the H^+ response in cells is contributed by interprotein interaction with spectrin, an inner membrane associated polypeptide. Recombination of spectrin with band 3 vesicles should provide the means for testing this hypothesis inasmuch as the former has been shown to confer pH induced aggregation properties to intralipid particles of reconstituted band 3 polypeptides.

In the alkaline range, the pH response of anion exchange is thought to be governed by the H^+ titration of critical membrane ligands of the transport mechanism. Our efforts are focussed on

identifying the critical ligands by specific chemical modification and labelling techniques of cells and isolated vesicles.

b. We are also purusing the possibility of isolating smaller functional segments of band 3 polypeptides, particularly those that are deeply embedded in the lipid matrix. We have so far succeeded in a 70% reduction of the original mass of band 3 by means of controlled proteolysis of vesicle preparations. These treatments apparently do not impair the anion exchange capacity of vesicles nor do they alter its susceptibility to specific inhibitors of anion transport. Isolation and reconstitution of the derivatized 20,000 fragment of the original 100,000 dalton protein should provide a unique tool for the projected chemical and physicochemical studies of transport at the molecular level.

Nucleoside transport

In order to expedite identification and isolation of nucleoside transport components by methods which we have already developed for anion transport systems, radioactive forms of the inhibitor APMI will be applied on cells. On the basis of our previous work with high affinity inhibitors, we have recently designed various affinity chromatography matrices which are now being tested for selective retention of nucleoside receptor protein in human red blood cell membrane fractions. We will use these techniques to isolate the putative transport components, reconstitute them into liposomes and thoroughly characterize them both in terms of their kinetic as well as chemical properties.

The comparison of chemical and kinetic properties of carrier systems for two distinctly different molecules, one a neutral nucleoside and the other a charged anionic species, is expected to give new insights into the various molecular mechanism that nature has selected for transporting molecules across membranes.

Publications

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11. Rothstein, A.: Knauf, P. A. and Cabantchik, Z. I.: Surface components of erythrocytes and their role in transport In "Surface properties of normal and transformed cells" R. O. Hynes, Editor. J. Wiley and sons, Sussex, England., 1977 (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF [illegible] RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01227-01 KE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Volume Regulation in Avian Erythrocytes

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

PI: Floyd M. Kregenow, LKEM/NHLBI

Other: Roderic E. Steele, Laboratory of Technical Development/ NHLBI

COOPERATING UNITS (if any)

Clifford S. Patlak, Chief, Theoretical Section of Statistics and Mathematics, Division of Biometry, Mental Health

LAB/BRANCH
Laboratory of Kidney & Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION
NHLBI/NIH, Bethesda, Maryland

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Hydrogen ions and an accompanying anion are produced as cells enlarge to reestablish their volume. As cells shrink to control their volume, hydioxyl is produced. The formation of hydrogen ions and hydroxyl correlate with initial shifts in cell water and net cation transport.

401

PHS-6040

OBJECTIVES

Studies from this laboratory, using avian erythrocytes as a model, originally identified and then characterized a transport mechanism capable of regulating cell size. This "volume controlling mechanism" returns cells to their original isotonic volume in either isotonic or anisotonic media by regulating the total number of osmotic particles within cells. It functions by controlling the movement of K into and out of the cell; the K, in turn, is accompanied by diffusable anion, primarily Cl, and finally osmotically obligated water. Two different transport processes function, depending upon whether the cells are in an enlarged or in a shrunken condition and thus need to either lose (cell shrinkage or gain (cell enlargement) cations.

As reported previously, during cell shrinkage or Volume Regulatory Decrease (VRD), the mechanism monitors K loss, in response to changes in cell size, by altering the resistance of a K pathway to K movement. The driving force for the K movement is simply the K electrochemical gradient.

On the other hand, the transport process involved in cell enlargement or Volume Regulatory Increase (VRI) was not as readily explicable. Cells enlarge not only in hypertonic media, consequent to the initial osmotic shrinkage, but also in isotonic media in response to catecholamines. Some progress has been made (see Annual Report 1975 & 1976) in characterizing this transport mechanism as a co-transport system in which Na and K are the co-transported species. The sum of the Na and K electrochemical potentials serves as the driving force for net movement. The summated potential, determines the direction and magnitude of the net cation flux. However, all hypotheses to date have inadequately explained why cell enlargement and net ouabain-insensitive cation uptake plateaus at $[K]_o$ of 15 mM and above. In pursuing this aspect of the problem, we found cells undergoing VRI underwent a pH change that correlated with $[K]_o$ in a way that suggested the pH change was involved in the co-transport process. This finding has led to our implicating a reaction involving the production and consumption of hydrogen ion and unknown anion in volume regulation.

MAJOR FINDINGS

A. 1 - As cells enlarge, they undergo a reaction that results in the production of hydrogen ions and an accompanying anions. The reaction does not depend on the nature of the initiating stimulus; it is similar when either norepinephrine or hypertonicity starts enlargement. The quantity of acid produced is large; nearly 10 times more acid is produced in 15 minutes than lactic acid is generated through the Emben-Meyeroff pathway.

2. Acid production correlates with cell enlargement. Like the enlargement process, it depends upon medium K concentration, increasing as the $[K]_o$ is raised from 2.5 mM to 15 mM and then plateauing as the $[K]_o$ is raised further. The rate at which acid is produced and cells enlarge is also similar. Like the volume changes, acid production is rapid initially and then gradually subsides. Similarly, the initial rate is more rapid if cells are stimulated with norepinephrine than with hypertonicity. Finally, agents or experimental procedures that inhibit enlargement, such as furosemide, replacing Cl with SO_4, or, in the case of norepinephrine, propranolol, inhibit acid production as well.

3. The initial hydrogen ion production correlates with the following three parameters of cell enlargement; 1) water uptake, 2) ouabain insensitive K uptake (slightly more than 3 cations per hydrogen ion).

4. Plant cells possess transport mechanisms that exchange hydrogen ions for Ca, Mg, or NH_4 while some bacteria exchange hydrogen ions for amino acids. We have eliminated the possibility that the hydrogen ion produced exchanges for any of these cations by directly measuring them in the extracellular medium. The possibility that hydrogen ion exchanges with K can be evaluated only if the rapid anion exchange system in red cells, which shuttles hydroxyl across the membrane, is first eliminated. Efforts to inhibit this anion transport system and still maintain the cell enlargement process intact have failed to date.

5. The identity of the anion remains unknown. It is not bicarbonate, however. We were able to evaluate bicarbonate production by using an instrument developed by Dr. Steele that measures nanomole quantities of bicarbonate. Although cellular CO_2 production and therefore bicarbonate formation is considerable, the difference between that of control and experimental cells is an order of magnitude too small to account for the hydrogen ion produced. The anion in question remains in the cellular compartment. The conclusion was reached, because of our inability to find a sufficient amount of any unassociated anion in the bathing medium. There are small quantities of lactate, pyruvate and phosphate produced in both experimental and control media, but their concentrations are not different in the two media.

B. 1 - When cells undergo cell shrinkage, a reaction similar in nature, but opposite in direction, occurs. Hydrogen ions and an accompanying anion are consumed and hydroxyl is produced. As before, the reaction develops whenever cells regulate their

volume; hydroxyl is produced in either hypotonic media or in isotonic media in which the cells have been previously enlarged by incorporating additional potassium salts.

2. The correlation between hydroxyl production and the quantity of either water or K lost is not good if the entire 45-60 minute response is considered. However, if the appraisal includes only the initial 15 minute response, the agreement between these two factors is impressive. One of the characteristic features of cell shrinkage is that there is an upper limit to the rate at which cells can lose K and water in response to an increase in cell volume. Thus, cells incubated in hypotonic media with an osmolality of 223 mosm. and enlarged 26% lost K and water at the same initial rate as cells incubated in a more hypotonic media, 188 mosm, and enlarged 40%. Hydroxyl production responds in a similar fashion. Both the quantity of hydroxyl produced in 15 minutes and the rate at which the system (cells plus medium) becomes alkaline increase as the medium osmolality is decreased to 223 mosm, but then the response plateaus as medium osmolality is decreased further to 188 mosm. Hydroxyl production during this period correlates with 1) water loss, and 2) ouabain-insensitive K loss which in this instance is equivalent to total cation loss (slightly more than 3 K per hydroxyl).

3. As is the case with cell enlargement, it is possible that hydrogen ions are transported exchange for K in cell shrinkage. In this instance both positively charged substances would be transported in a direction opposite to that proposed for cell enlargement. Efforts to evaluate this possibility have been unsuccessful to date.

C. In collaboration with Dr. C. Patlak, plans are being formulated to model the volume controlling mechanism and to examine certain aspects of the hypotonic response.

FUTURE COURSE

1. To identify the anion produced in cell enlargement and consumed in cell shrinkage.

2. To decipher whether hydrogen or hydroxyl production correlates with initial 1) water changes, 2) net ouabain insensitive K influx, or 3) total net cation movement.

3. To further evaluate the role these reactions play in volume regulation.

PUBLICATIONS

1. Kregenow, F. M.: Cell Volume Control in Water Relations in Membrane Transport in Plants and Animals, eds. A. M. Jungreis, T. K. Hodges, A. Kleinzeller, and S. G. Schultz, Academic Press, New York, 1977, pp. 291-302.

2. Kregenow, F. M.: Transport in avian erythrocytes in Transport in Red Cells . ed. Clive Ellory, Academic Press, New York, 1977, pp. 383-426.

3. Kregenow, F. M.: An assessment of the co-transport hypothesis as it applies to the norepinephrine and hypertonic responses in Osomtic and Volume Regulation, The Alfred Benzon Symposium XI, eds. E. Skadlauge amd C. B. Jorgenson, (in process, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01228-01 KE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Volume Regulation in Avian and Reptilian Erythrocytes

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

PI: Arthur Siebens, Guest Worker, LKEM/NHLBI

Other: Floyd M. Kregenow, Sr. Investigator, LKEM/NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Kidney & Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION
NHLBI/NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4/12	1/12	3/12

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)

A possible role of calcium as an intracellular mediator of volume regulation is being investigated using A23187 and specific pharmacological agents. The transport mechanisms responsible for volume regulation in Amphiuma red cells are being studied in preparation for their use in a single cell micropuncture technique.

OBJECTIVES

The purpose of this project is to study the role of cellular calcium in volume regulation and in particular in cell shrinkage. In other erythrocytes, calcium causes a non-physiological release of potassium which produces an uncontrolled shrinkage. During cell shrinkage duck erythrocytes release potassium physiologically so as to shrink and correct their volume. We hope to implicate calcium as a mediator of the physiological response by first establishing the non-physiological response in duck erythrocytes, using A23187, and then comparing the K efflux characteristics of both systems.

Numerous inhibitors of the non-physiological response will be used to investigate the possibility that a common potassium efflux pathway responds to both physiological and non-physiological stimuli. Discovery of a common blocking agent would not only suggest that calcium serves as intracellular mediator of the physiological response, but would also provide a useful tool for studying cell shrinkage.

A future goal involves the study of volume regulation in a single cell using a micropuncture technique developed in this laboratory for the giant red cell of the salamander, Amphiuma. Since the volume controlling mechanism has not been adequately demonstrated in Amphiuma red cells, we are currently studying populations of packed Amphiuma red cells to verify that they have this ability.

MAJOR FINDINGS

1. The calcium ionophore A23187 produces a 2-3 fold increase in potassium permeability at a $(Ca)_o$ of .5 mM and an ionophore concentration of 4×10^{-5} M. Testing of agents which block increased K effluxes will begin shortly.

2. Amphiuma red cells appear to be able to regulate their volume in hypotonic media. However, the response takes several hours, unlike that in duck red cells. Optimal buffer conditions are currently being worked out for the prolonged incubation.

PUBLICATIONS - None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01229-01 KE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Hormonal Control of Cation Transport in Avian Erythrocytes

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

PI: Floyd M. Kregenow, Sr. Investigator, LKEM/NHLBI

Other: None

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Kidney & Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION
NHLBI/NIH, Bethesda, Md.

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)

Certain synthetic catecholamines were studied for their ability to initiate the specific cation transport mechanism responsible for volume regulation. Effective compounds were found to act at a specific receptor site and to be structurally similar to two amino acids.

OBJECTIVES

Norepinephrine-induced enlargement affords an excellent opportunity to study a hormonally-induced transport system in its entirety. The system can be divided into two parts: 1) transport and 2) those events associated with hormonal induction. This laboratory has made some progress in characterizing the former as a co-transport process in which Na and K are the co-transported species. It is generally accepted that the latter involves a series of events that begins with hormonal recognition by elements on the external surface of the membrane. Subsequently, adenyl cyclase is activated and cyclic AMP formed. This laboratory has shown previously that the latter acts as a messenger to effect a cellular event that then initiates the transport process.

Our interest in hormonal recognition is two-fold. First, it represents an example of the more general problem of how cells receive information from their environment. Second, we know from previous studies that whatever the hormone and cyclic AMP eventually do, their effect can be mimicked, in a way that bypasses the recognition step, by simply placing cells in hypertonic media and shrinking them.

The structures of norepinephrine and epinephrine resemble that of the amino acid phenylalanine from which they are synthesized. The catecholamines lack α carboxyl group that is present on the amino acid, but retain the α amino group and possess, in addition, 3 hydroxyl groups. A hydroxyl group on the β carbon and the 3rd position of the ring structure accounts for two of the hydroxyls. The third hydroxyl is located on the fourth position of the ring structure and is a normal constituent of the amino acid tyrosine.

Hormone recognition can be viewed as a lock and key phenomenon. The key (hormone) must fit a lock (receptor) in a way that causes a specific response. Since the amino acids phenylalanine and tyrosine do not activate VRI even at concentrations as high as 10^{-2}M, whereas norepinephrine and epinephrine are effective at concentrations as low as 10^{-8}M, some or all of the structural differences mentioned above are necessary for "normal" hormonal recognition. By using catecholamine analogues with a single structural difference, one can deduce what effect each structural change has on the "normal" recognition response.

METHODS

We measured K influxes and determined specificity by noting their responses to the beta-adrenergic blocking agent, propranolol.

MAJOR FINDINGS

1. The α amino group and its associated positive charge appears to be essential. Compounds similar to epinephrine and norepinephrine which contain a hydroxyl instead of the α amino group (3,4 dihydroxyphenylglycol, 3,4 dihydroxy-mandelic acid) fail to act at concentrations as high as 10^{-2} M.

2. The addition of a side chain on the α amino group may enhance sensitivity; isoproterenol with a $CH_3(CH_3)_2$ is more effective at 10^{-9} M than norepinephrine which lacks a side chain. However, the presence of a side chain does not assure enhanced sensitivity; epinephrine with a CH_3 group is less effective than norepinephrine at a concentration of 10^{-8} M.

3. The continued presence of the α carboxyl group and its associated negativity is inhibitory. D, L threodihydroxyl-phenylserine, which otherwise is like norepinephrine, still activates, but only at concentrations of 10^{-4} M and above.

4. Removing any one of the three hydroxyl groups produces a class of compounds with similar effects. Compounds with a hydroxyl group on only the β carbon and third position (norphenylephrine and L-phenylephrine), β carbon and fourth position (DL-octopamine and DL synephrine) or the third and fourth position (dopamine) are like norepinephrine and epinephrine in that they activate VRI, but differ in that the concentrations needed to produce a significant response are three orders of magnitude larger (10^{-3} M). And, once activated, a maximum response requires a 10-fold larger concentration than is necessary with norepinephrine and epinephrine. Volume regulation though is similar. Cells gain K, Cl, and water in a $[K]_o$ dependent fashion and demonstrate the same dramatic increase in both Na and K fluxes.

5. If two hydroxyl groups are removed, only compounds with a hydroxyl group remaining on the β position can initiate VRI. A 1 mM concentration of β-hydroxyl phenethylamine produces 10-30% of the maximal norepinephrine response.

6. Compounds lacking all three hydroxyls are inactive (DL-phenethylamine).

7. Findings 2 through 6 indicate that the removal of the α carboxyl and the simultaneous addition of hydroxyl groups are the most important structural changes when a comparison is made between the catecholamine and the amino acid phenylalanine. Of the three hydroxyls, the one in the β position appears to be the most effective, but it is not essential. With the addition of each hydroxyl, a more sensitive compound is produced. Not

until all three hydroxyl groups are present, does one have a "true hormone" capable of acting in the nanomole range.

8. The preceding analysis depends, in part, upon the belief that all active compounds interact with a single receptor site (s). The available evidence favors this view. First, propranol, a beta-adrenergic blocking agent, inhibits all active compounds at the same low concentration irregardless of the class of compounds or the concentration needed to activate. Second, the same maximal response is produced by all compounds containing either two or three hydroxyl groups. Furthermore, maximal responses by these compounds are not additive. Third, if the return of fluxes to normal as cells enlarge can be considered to represent a state of "receptor inactivation," then the fact that neither class of compounds (i.e. those containing either two or three hydroxyl groups) can restimulate cells, previously activated by the other class, suggests that an identical receptor is involved. Not supportive, however, is the fact that when compounds from more than one class are incubated together at concentrations that would normally yield submaximal responses (<50%), they produce additive effects.

9. If there is a single receptor site, then it follows, since the hormonal response appears to have had an amino acid-like evolution, that the process of hormonal recognition may have similarities to the way membranes recognize amino acids.

FUTURE COURSE

1. To determine whether the response of those compounds that activate VRI but have only one or two hydroxyl groups involves the synthesis of cyclic AMP.

2. To develop a system for following the maturation of the hormonal recognition process during erythrocyte differentiation.

PUBLICATIONS None

ANNUAL REPORT OF THE
LABORATORY OF TECHNICAL DEVELOPMENT
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
JULY 1, 1976 to SEPTEMBER 30, 1977

This laboratory continues to develop new instruments and methods to facilitate medical research, diagnosis or therapy. In many respects, the boundaries of our knowledge and our ability to apply what we know are limited by our technology. Methods development and new instrumentation serves to widen our horizons and to convert knowledge to practice. We have a group of scientists selected for their interests and capabilities to identify requirements of biomedical science that can benefit from the application of instrumentation science and technology. We develop working systems that can be applied at NIH in conjunction with ongoing research activities,make the results available to the scientific community and industry by publishing the method or instrument. The areas of our activity include analytical methods and separation techniques, not only for chemicals but also for living cells; clinical applications include regional blood flow measurement, materials and methods development for better cardiopulmonary support for both isolated organs and whole animals.

The Section on Biophysical Instrumentation established this past year is developing instruments for the study of enzyme protein interaction. The section is currently engaged in assembling a group of instrumental methods for the study of the hemoglobin oxygen complex in collaboration with the Clinical Hematology Branch.

While the general direction and the selection of the technology required remains in the section,the engineering development and construction of specific components is delegated to the BEIB and occasionally to industry to most economically utilize available technology. The advantages of this interaction with the Engineering Branch is acknowledged.

An apparatus has been developed for the continuous measurement of the dissociation curve from less than .5mm Hg oxygen tension to 1 atmosphere. Such a curve takes only 15 minutes on a .5 ml sample of whole blood. It is well known that the P50 may be invariant to DPG concentrations in different patients, but not necessarily in the same patient. Of particular interest is how the DPG and various therapia such as cynate affect the whole course, not just the P50, and a clearer understanding of this is necessary for proper therapy of the sickle cell or other hemoglobinopathy patient. The success of this instrument was such that the amount of data generated required some sort of computer data handling. The first stage was to provide a storage device and a graphic terminal which connected to our large PDP-10. Recognizing the eventual high cost of using the PDP-10, BEIB developed, under our direction, a microprocessor system which runs the experiment, does the evaluations, and draws the graphics. Output goes to the mass storage device.

A liquid dye laser flash photolysis system has been developed primarily for the study of oxygen binding to hemoglobin, though of course it is applicable to any reaction capable of being initiated by a short intense light pulse. At present the system is limited by the detection system to dilute solutions, 1 mM or less Hb. In collaboration with Electronic Engineering, BEIB, a detection system using near infra-red wavelengths is being developed which consists of two light emitting diodes and a pair of phototransistors. This scheme should extend the range of the photolysis system to higher concentrations and allow study of reaction kinetics inside the red blood cell.

The high speed optical stopped flow apparatus has been modified to allow combined stopped flow-laser photolysis studies to be carried out. This arrangement is suitable for investigating reaction where a reaction intermediate can be formed using the flow apparatus and the laser pulse can then be used to interrogate this intermediate.

A Mossbauer facility has been assembled to study its applicability to the study of hemoglobin states. The Mossbauer Effect (ME) is the recoilless emission and subsequent recoilless absorption of gamma radiation. The importance of the ME to the investigation of biological processes lies in the fact that it involves radiation of the highest energy resolution available. This allows us to measure very small energy differences ($\sim 10^{-9}$ eV). Because the absorption cross section of this radiation in the Mossbauer isotope is six orders of magnitude greater than the next competitive process we can perform measurements on small amounts of material ($\sim 10^{12}$ atoms). The effect can take place only under special conditions in about 100 isotopes. The only isotope of iron that can be utilized in Mossbauer work is Fe^{57} which has a natural abundance of 2.17%. Isotopic enrichment therefore, plays an important role in these experiments. The Mossbauer effect has previously been used to study heme proteins. The important information that can be obtained from the Mossbauer effect, for our purposes, is the rather detailed knowledge of the state of the iron ion. Because the environment of the ion changes during biological and chemical processes and the state of the iron does likewise these changes are reflected in the Mossbauer spectrum.

Fast, reliable thermistors developed for use in the study of biochemical reactions have now been thoroughly tested and two types are now available commercially. A new differential amplifier developed to be used with them has been incorporated into prototypes of two new instruments. One, a differential thermal titration calorimeter is to be used in conjunction with a new differential pH meter, to measure simultaneously the thermometric and potentiometric titration curve of a protein. The complete curve, from pH 4 to 10 can be run in 2 minutes on a 2 ml sample containing 0.1 μ mole of protein. Such titrations permit the protein chemist to identify the groups active in biochemical reactions as well as those reacting with inhibitors.

The spiral coil membrane lung developed by the Section on Pulmonary and Cardiac Assist Devices continues to hold its high position for atraumatic pulmonary support by extracorporeal gas exchange. If the membrane lung is

used primarily for oxygenation, then the full cardiac output must be obtained and the procedure is accompanied with hazards of prolonged high volume extracorporeal circulation. On the other hand, if the membrane lung is used primarily for CO_2 removal, only a small fraction of the cardiac output need be passed through the extracorporeal circuit. This is because the CO_2 capacity of blood is several-fold more than for O_2. Optimization of the membrane lung for CO_2 removal has led to a device which could reduce the burden on the patient substantially. Extracorporeal CO_2 removal from lambs has shown adequate oxygenation in the absence of mechanical or natural ventilation, with only 5 cm Hg of positive pressure of O_2 delivered to paralyzed lungs. Lung mechanics have been studied at various sub-normal ventilatory rates for periods of more than 24 hours, and have shown no deliterious effects. The possibility that this form of support could provide advantages over mechanical respirators is being evaluated.

Incidental to above studies the pulsation of blood in the pulmonary circuit was examined with a high sensitivity pulmonary plethsymograph developed for the study. The new plethsymograph is about 100 times more sensitive than the apparatus used in previous studies.

When we used a commercial blood separator in some previous experiments we noted that the separator produced cumulative damage to the blood, presumably due to the rotating seals in the centrifuge. We therefore decided to see if we could improve the method by using a scheme that we used previously for a chemical separation instrument that obviates the need for rotating suface seals. In addition, we exploited our experience and capability in silicone rubber fabrication and cast a centrifugal separator rotor of new design. In the new system the blood only contacts silicone rubber in a narrow channel. It separated plasma, red cells, lymphocytes, granulocytes, and platelets at flows up to 400 ml/min. This development is continuing.

Continuing developments in countercurrent chromatography have now shown that a relatively simple helical column with a two phase solvent system slowly rotating in the normal gravitational field can be applied on a preparative scale (10 ml). Amino acid separations were used to provide comparative performance data. Efficiency and freedom from support complications make this system potentially useful.

Another arrangement of a preparative scale helical column was developed and tested which used both gravitational and centrifugal force fields for performing the two phase solvent separations in much shorter times than with the simple gravitation field system. The two systems have been thoroughly described and publications give details for application to biomedical research.

The system for monitoring blood PO_2 and PCO_2, which had already shown the ability to perform for 24 hour periods, has been improved substantially. Causes of drift, premature loss of sensitivity, and component failures have been identified and corrected. These improvements have made the

unit considerably more reliable and, therefore, useful. A microprocessor controller now makes the computations needed to convert the transducer outputs to a digital display, corrects for temperature effects, and controls the operation of the system. In addition to the flow-through probe used with extracorporeal procedures, a catheter probe is being tested. This probe, less than 1 mm outisde diameter, could be used in situations where frequent blood-gas measurements are needed by an extracorporeal circuit is not used.

The use of fluorescent dyes as probes of protein and membrane structure has been examined in several areas:

1. The properties and interactions of Auramine O, a cationic dye, have been measured. Its interation with serum albumins and alcohol dehydrogenases serve as a model for studies of cationic ligands with proteins, and it was shown that the binding is generally weaker than with anionic dyes, but of interest since the cationic dye probes different sites.

2. The interaction of diphenylhexatriene, or DPH, with proteins was studied with fluorescence spectroscopy and fluorescence decay kinetics. The dye has been assumed to bind mainly to lipids of cell membranes, but our study shows DPH to be a probe of protein structure and protein-lipid interaction.

3. Red cell membrane fluidity has been studied with DPH as a probe, in a study of the effect of lipid feeding in dogs undergoing experimental atherosclerosis.

A general fluorescence method for certain types of lipolytic enzymes has been developed. A concentrated solution of a dye is encapsulated in lecithin liposomes about 300 Å in diameter. The dye is largely self-quenched until released from the liposomes by lecithinases such as phospholipase A. The amount of dye released is proportional to the enzyme concentration. Extension of this method, "fluorescence quenching release", to other enzymes is in progress.

The construction of the instrument to measure changes in cerebral vascular perfusion with niether interference from overlying tissue nor harmful radiation or added intravascular tracers is continuing. In this laboratory the electronic circuits for depolarizing and sensing the protons that carry the signal in the blood have been refined and tested on model flow circuits. The problem of discriminating the signal of the relatively few protons in the blood vessel from those in the mass of tissue overlying the vessel has been resolved. The use of a weak field low frequency detector reduces the susceptibility of local protons but prepolarized protons that have traversed the intracranial volume that is being measured have residual prepolarization that contribute to a stronger signal and that prepolarization is modulated at a frequency that aids in distinguishing it from noise. In the course of this development special coil configurations and the

associated coupling circuits were developed. These coils differ from the usual nuclear magnetic resonance coils in that the sample cannot be enclosed in the coil as in the usual chemical NMR methods.

The application of the instrument to animal tests is being carried out at the Medical College of Wisconsin under contract to this laboratory. They are providing facilities for small animal and primate studies and ultimately human studies. With our support they have constructed several model instruments and validated the idea in several modes of operation and are currently installing a large superconducting solenoid that will produce high magnetic fields of 2T to provide a great improvement in sensitivity by producing an intense prepolarizing field that will greatly improve the sensitivity and facilitate the measurements. A calculated improvement of some 80 times in signal strength is expected. The design and construction of coils and receivers is nearly complete and the combined system is expected to be functional soon.

PROJECT NUMBER (Do NOT use this space)	HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	Z01 HL 01404-09 LTD

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Membrane Lung Systems for Long Term Support

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

PI:	T. Kolobow	Chief, Section on Pulmonary & Cardiac Assist Devices	LTD NHLBI
OTHERS:	L. Gattinoni	Visiting Fellow	LTD NHLBI
	D. White	Associate	LTD NHLBI
	T. Tomlinson	Biologist	LTD NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Technical Development

SECTION

Section on Pulmonary and Cardiac Assist Devices

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3-1/4	2-1/4	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)

It is the goal of this project to develop an improved membrane lung suitable for long-term pulmonary and cardiopulmonary support. Current activities center around: 1. applying carbon dioxide elimination techniques to specific experimental situations for pulmonary assist, 2. developing a carbon dioxide membrane lung for high efficiency carbon dioxide elimination at low blood flows, 3. improving blood compatibility of membranes for use in the membrane lung. We are investigating the advantages of carbon containing membrane for improved blood compatibility, 4. investigating the nature of pulmonary capillary blood flow, factors affecting capillary blood flow, and means to alter pulmonary capillary blood flow.

PHS-6040
(Rev. 10-76)

Objectives:

1. We are continuing to document the advantages and safety of the membrane artificial lung both in short term and for long term use. At present, open heart procedures are primarily performed with bubble oxygenators, which cannot safely be used beyond 4-6 hours. It is desirable to develop an artificial lung (blood oxygenator) which could safely be used during even the most prolonged open heart surgical procedure encountered i.e. over 6 hours.

There is a great potential for the use of artificial lung systems in the treatment of various primary pulmonary disease processes as acute respiratory failure, as well as in chronic respiratory failure. Such a system in all likelihood will be based on the membrane lung, which has consistently out performed all other methods for long term extracorporeal respiratory blood gas exchange.

2. The major problem in artificial internal organ research is blood compatibility. This problem is magnified in devices having a large surface area, such as the artificial kidney and the artificial lung, although it is also important in implantable devices such as heart valves, synthetic blood vessels, etc. At present heparin anticoagulation must be used during all procedures using the artificial lung, and the artificial kidney.

Our goal is to improve blood compatibility of membranes used in the membrane artificial lung, and not impair permeability to respiratory gases.

3. The artificial lung (whether used in open heart surgery or during long term application for acute respiratory failure) is usually referred to as a blood oxygenator-ignoring its equally important function of CO_2 elimination. The fact that the resting carbon dioxide production can be removed by passing 500-1000 ml/min of blood through an efficient membrane lung opens the possibility of removing extracorporealy all carbon dioxide produced in man, and thereby substantially modify spontaneous or mechanical pulmonary ventilation.

Using animal experiments, we are investigating the possibility of selectively removing carbon dioxide in patients who could reasonably benefit from extracorporeal carbon dioxide removal. This patient population includes patients ventilated with mechanical pulmonary ventilators in whom it may be desirable to lower peak inspiratory pressure to prevent barotrauma. It is also possible that extracorporeal carbon dioxide removal may obviate the use of a mechanical ventilator.

Similarly, patients with chronic obstructive pulmonary disease with carbon dioxide retention can have $PaCO_2$ restored to a physiological

range through an extracorporeal membrane lung, and thereby substantially improve pulmonary ventilation. We do not expect that chronic obstructive pulmonary disease can be cured; rather, we visualize a treatment protocol similar to artificial kidney use, i.e. once weekly or even monthly CO_2 washout by a membrane lung.

Methods Employed and Major Findings:

1. Blood compatibility studies.

Membranes produced in this laboratory have undergone animal testing by the arteriovenous shunt technique. In this method, the test membranes were made into completed SCML and evaluated under actual operating conditions. All spiral coiled membrane lungs (SCML) were primed by the CO_2 priming technique.

We measured changes in the resistance to blow flow, changes in blood platelet count, white blood cell count, and differential count. Lack of change in all the above parameters implied excellent blood compatibility. No heparin was given once bypass was begun.

In a previous study, we have shown that silicone rubber membranes made by the two layer casting technique, with the blood contacting layer containing polydimethylsiloxane (PDMS) showed the best blood compatibility over other commercially available silicone polymers.

All synthetic membranes used in membrane lungs, or the artificial kidney, have uniformly caused marked granulocytopenia and leukopenia during the first 5-15 min. of initiating bypass-except for one silicone rubber membrane modified by the addition of some acetylene black carbon. This membrane had been prepared by a three layer casting technique: the first layer was standard silicone rubber containing silica filler (30%), the second layer was polydimethylvinylsiloxane enriched with 25% acetylene black carbon, and a third (blood contacting side) was made of standard silicone rubber with 30% of silica filler. We have now cast 3 layer silicone rubber membranes where the third layer in the membrane was standard silicone rubber with only 0.1% and 0.3% silica filler, and tested this material in a completed spiral coiled membrane lung in an A-V shunt in lambs.

Our results showed that WBC effect was not maintained when the concentration of silica filler was reduced to 0.1 and 0.3% and that the membrane was in fact rather thrombogenic. Studies exploring the higher range of silica filler in the third layer of the membrane are now in progress.

2. The Carbon Dioxide Membrane Lung (CDML).

Membrane pinholes have been the major problem that have plagued commercial producers of membrane lungs. These problems have been solved by the double layer casting technique developed in this laboratory. It is routine to cast silicone rubber membranes with a thickness of 36 to 44 micrometers without defects.

The development of high efficiency CDML (a device optimized for carbon dioxide removal) requires a low perfusion pressure and an exceptionally high carbon dioxide transfer rate. The CDML developed in this laboratory contains the following features:

a. The CDML silicone membrane is made of a very thin membrane (36 micrometers) for enhanced carbon dioxide diffusion.

b. The perfusion pressure in the CDML is low (50 mm. Hg/1000 ml blood flow per minute) so as to allow the CDML to be operated without a blood pump, i.e. under arterial pressure head alone. We have accomplished this by embossing the spacer screen of the CDML. The net effect of this has been a substantial reduction of blood flow resistance and an increase in carbon dioxide exchange rate to 90 ml/m2/min.

Animal Studies:

Extracorporeal carbon dioxide removal markedly affects spontaneous breathing, and also mechanical pulmonary ventilation. In studies in lambs, various amounts of carbon dioxide were removed by extracorporeal carbon dioxide membrane lung (CDML). We measured response of extracorporeal CO_2 removal on spontaneous breathing, and to various methods of mechanical pulmonary ventilation:

1. Control of breathing through extracorporeal carbon dioxide removal by the CDML.

In these studies, progressively increasing fractions of CO_2 were removed by the CDML and the animal adjusted its breathing to meet normal physiologic requirements.

We found that spontaneous alveolar ventilation decreased inversely with increase in extracorporeal CO_2 removal. For example, when 60% of produced CO_2 was removed by the CDML, alveolar ventilation also decreased by 60%; when virtually all CO_2 was removed extracorporeally, spontaneous breathing virtually ceased.

We belive these findings have direct clinical relevance in the management of patients with CO_2 retention, or in whom it is desirable to reduce the work of breathing.

2. Control of mechanical ventilation with extracorporeal removal of CO_2

by the CDML.

It follows from (1) that when progressively greater amounts of CO_2 are removed by the extracorporeal CDML, alveolar ventilation can be similarly reduced. In animal studies, we have now shown that when progressively increasing fractions of CO_2 are removed by an extracorporeal CDML, mechanical alveolar ventilation can be reduced by the same product.

This finding has great practical significance in patient management with "stiff lungs" in whom nowadays particularly high airway pressures must be used to accomplish "adequate pulmonary ventilation".

3. Low Frequency Positive Pressure Ventilation (LFPPV) and extracorporeal CO_2 removal. A new concept.

Much is kown about deleterious side effects of postive pressure ventilation on decreased function of internal organs "below the diaphragm"-i.e. the liver, kidneys, pancreas, etc. At times, death of the patient on a mechanical ventilator does not stem from decreased respiratory gas exchange through the lungs, but from multiple organ dysfunction, part of which can be directly ascribed to the use of positive pressure ventilation.

In a series of lamb studies we provided for substantially total removal of all produced CO_2 by an extracorporeal CDML and then ventilated the lambs with a mechanical ventilator at a rate of 0.66 to 4 breaths/min, at a tidal volume (TV) of 3, 10, and 15 ml/kg. We found that the total lung compliance, functional residual capacity (FRC), and venous admixture were substantially better at TV 15 ventilation at all respiratory rates studied, including at a frequency of only one breath every 90 seconds.

We know that significant impairment in FRC, lung compliance and venous admixture occurs in animals ventilated for 12 hours in the conventional fashion. In contrast, with LFPPV and extracorporeal CO_2 removal we find either no change, or in fact improvement in lung function after twelve hours of LFPPV! The latter improvement is based on measurements before bypass, and is relative to baseline values after anesthesia and surgery.

4. Apenic oxygenation.

From the previous it is clear that when all CO_2 produced by the animals is removed by the CDML, breathing will cease and the animal will die from anoxia in spite of presumably a continuing hypoxic stimulus to breathe. However, if 100% oxygen is continuously supplied though a small catheter at a rate equal to his oxygen consumption, then alveolar oxygen concentration will remain unchanged and blood oxygenation will continue unimpaired.

We have sedated and paralyzed young lambs and removed all produced CO_2 by an extracorporeal CDML. When oxygen was fed directly into the trachea in a quantity equal to oxygen consumption of the sheep, arterial blood PO_2 remained normal for the duration of the study. After 24 hours of apnea, these animals were then allowed to recover from anesthesia and paralysis. There was no deterioration in FRC, blood gases, or total lung compliance. In our hands the procedure appears to be particularly safe, and easy to use.

We have shown in this study that prolonged apnea (one to 3 days) is entirely feasible if CO_2 is removed extracorporeally by a CDML.

It is not that we advocate apnea, the cessation of all breathing for the treatment of any pulmonary disorder. Rather, we wish to point out the great variability that can be introduced in the management of patients with respiratory problems using mechanical pulmonary ventilators-or possibly even in avoiding the use of a mechanical pulmonary ventilator.

5. A common result of deteriorating pulmonary status in patients with acute respiratory failure is manifested in increase in venous admixture. We have hypothesized that capillary blood flow is altered during acute respiratory failure; if so, ways may be found to mechanically or pharmacologically to re-direct pulmonary capillary blood flow and thereby perhaps to reduce pulmonary venous admixture.

It was shown some 20 years ago using total body plethysmography that pulmonary capillary blood flow is pulsatile. Many studies since then appear to have confirmed these findings. Our studies using techniques substantially more accurate than used in the previous studies show that pulmonary capillary blood flow is, instead, nonpulsatile, and is therefore same as the capillary blood flow in the systemic circulation.

We have constructed a highly sensitive volume transducer using a diaphragm 10 cm in diameter, 20 micrometers thick, made of silicone rubber and a position transducer based on eddy current principle in a bridge circuit. Studies were performed in a total body plethysmograph. The resolution of the entire system was better than 10 microliters out of a total plethysmograph volume of 100 liters, and the frequency response of the entire system was almost flat to 30 cps.

All studies were done during "apneic oxygenation" with extracorporeal CO_2 removal. Tracer gases consisting of 10 to 50 ml each of oxygen, carbon dioxide, nitrogen, nitrous oxide were injected directly into the trachea through a small catheter and changes in volume in the plethysmograph was measured and recorded. Our results showed that uptake of oxygen, nitrous oxide, and carbon dioxide was continuous at normal heart rates. As expected, there was only a barely perceptible

uptake of nitrogen after the small change in PN_2 in the alveolar gas. It follows that pulmonary capillary blood flow is continuous, and not pulsatile. During extreme bradycardia (heart rate less than 30/min) the pulmonary capillary blood flow stops during late diastole.

The finding of nonpulsatile pulmonary capillary blood flow comes perhaps as no surprise as the capillary blood flow in the systemic circulation is nonpulsatile under ordinary circumstances.

We do not know whether or how pulmonary capillary blood flow is altered in disease states. This knowledge is a prerequisite to attempts at altering pulmonary capillary blood flow by mechanical, or pharmacological means.

Significance to Biomedical Research and the Program of the Institute:

The need for an improved artificial lung is found in open heart surgery where blood trauma can be substantially reduced by using more gentle forms of respiratory gas exchange. This will reduce the need for blood transfusion postoperatively, and reduce morbidity and mortality.

Of all types of artificial lungs in clinical use, the membrane lung is by far the most benign for human applications.

In the area of pulmonary support, only the membrane lung is a likely candidate for possible use. The demands in long term membrane lung application are substantially more severe than in short term surgical procedure. To be of practical use, an extracorporeal membrane lung system cannot adversely affect blood protein or blood cellular components beyond the body's ability to compensate. Ideally, the entire membrane lung and perfusion system should be operated without the need for systemic anticoagulation. Our present effort in formulating silicone rubber membranes with improved blood compatibility will, we believe, extend the safe period of longterm support with the membrane lung for acute, and chronic respiratory failure.

Our laboratory work has shown that breathing can be controlled by extracorporeal carbon dioxide removal with the CDML. This has opened the possibility, for the first time, to effectively modify mechanical pulmonary ventilation without regard to limitations imposed by "adequate ventilation" as practiced until now.

We have found pulmonary capillary blood flow to be non-pulsatile in nature. This finding was obtained with instrumentation and techniques substantially more accurate than previously available. Our results can help to further elucidate abonormalities in pulmonary capillary blood flow in health and disease.

Proposed Course:

1. We will continue to explore blood compatibility of carbon containing silicone rubber membranes for possible use in the membrane lung.

2. A program to develop a novel type of membrane lung suitable for long term implantation will be continued. This will include the preparation of new types of surfaces of membranes, and new designs to make a reliable compact unit.

3. The CDML will further be optimized for performance. Effort will be made so it can be applied directly to a patient through an A-V shunt or A-V fistula, analogous to artificial kidney use today.

Publications:

1. Gattinoni, L., and Kolobow, T.: "Respiratory quotient during dialysis. NEJM. 297: 56, 1977.

2. Kolobow, T., Gattinoni, L., Tomlinson, T., and Pierce, J.: "Control of breathing using an extracorporeal membrane lung. Anesthesiology. 46: 138-141, 1977.

3. Kolobow, T., Tomlinson, T., and Pierce, J.: "Blood compatibility of methyl, methyl vinyl, methyl phenyl, and trifluoropropylmethylvinyl silicone rubber without silica fillers in the spiral-coiled membrane lung. J.Biomed. Mater. Res.. 11: 471-481, 1977.

4. Kolobow, T., and Tomlinson, T.: "Vacuum priming of the membrane lung: a new and rapid technique. CVP. March/April, 1977.

5. Suaudeau, J., and Kolobow, T.: "Fresh platelet-rich plasma from a flow-through centrifuge for lamb heart perfusion at 13°C. J. Thoracic & Cardiorasc. Surg.. 72: 769-777, 1976.

6. Suaudeau, J., and Kolobow, T.: "Isolated sheep heart hypothermic (5-13° C) perfusion with fresh blood: successful preservation for 24-72 hours with continuous strong ventricular activity. Cryobiology. 14: 337-348, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZOL HL 01407-14 LTD

PERIOD COVERED
July 1, 1976 to September 30, 1977

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

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Technical Development

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INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	.6	.6

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The purpose of this project is to use fluorescence and/or phosphorescence methods to investigate problems of interest in biomedical areas. Recent work has included: 1. Study of cationic fluorescent dyes such as Auramine O, as fluorescent probes of proteins. Serum albumin and alcohol dehydrogenases were studied. 2. Study of DPH, diphenylhexatriene, a fluorescent probe which has an affinity for lipids and hydrophobic areas of proteins. 3. The study of red cell membrane fluidity, and the effect of lipid feeding on this parameter in dogs undergoing experimental atherosclerosis.

Objectives:

The aim of this project is to apply techniques and methods of fluorescence and phosphorescence spectroscopy which were previously developed in this laboratory to problems of current interest in biochemistry and related fields. By constant testing and application of such methodology, it is possible to refine the procedures and to make other workers more aware of their value.

Methods Employed:

The characterization of the fluorescence of solutions was carried out with spectrofluorometers, lifetime apparatuses, and spectrophotometers already present in the laboratory, some of which were described in previous publications. The materials examined, such as proteins, were obtained commercially. Where some collaboration with other laboratories took place, materials supplied by the collaborating laboratory was utilized.

Major Findings:

Our previous study on the properties of Auramine O, a cationic dye, have been published. We studied this dye becuase it had been reported to be one of the very few cationic fluorescent probes capable of forming fluorescent complexes with proteins. Most such probes are anionic, and seem to attach to the active site of many enzymes. Our study showed that Auramine O complexed with yeast alcohol dehydrogenase and several serum albumins of different species. The general conclusion was that Auramine O binding was weaker, as shown by equilibrium dialysis and fluorescence enhancement titration, than that of most anionic probes; and that this cationic dye was bound to sites other than those which bound the anionic probes. In order to probe such secondary topography of enzymes, investigators should expect to use higher concentrations of a cationic fluorescent probe than is usually used for anionic probes. Some additional data, such as the interaction of alcohol dehydrogenases with other cationic dyes, are still unpublished as of now, but may be integrated into another study at a later date.

We have examined the properties of DPH, diphenylhexatriene, which is a fluorescent compound used by others as a probe of lipid environments. This compound is said to be selective for the lipid portion of lipoproteins or cell membranes, and has been used to measure the viscosity of such enviornments by fluorescence polarization. We examined DPH binding to proteins, because we felt that the assumption that the compound bound only to lipids had not been established. We have found: 1. DPH does bind to proteins such as serum albumins, beta-lactoglobulin, ovalbumin, pepsin, etc., to give fluorescent complexes. By measuring the polarization one can obtain relaxation times in the usual manner. 2. The binding to serum albumins has been characterized by measuring the emission spectra, lifetimes, and quantum yields. 3. When fatty acids are added to serum albumins, they are known to

be tightly bound. The DPH-albumin complex is greatly altered by addition of fatty acids, and the spectra, quantum yields, and lability towards photodegradation are all changed. The data are consistent with the migration of DPH from a protein environment towards the lipid environment. It is possible tht hydrocarbons are carried in the lipid "pocket" formed by fatty acids bound to serum albumins. 4. DPH is also a good probe of micelle formation, and the critical micelle concentration of detergents like sodium lauryl sulfate and cetylammonium bromide determined with DPH as a probe agree with those in the literature. A paper is being prepared to present these results.

Collaboration with several other laboratories has occupied some of our time.

1. Our previous study on a new fluorescent probe, N 105, developed by Walter Steward of NIAMDD was written up and is soon to be submitted for publication.

2. Dr. Gullino, NCI, is engaged in a project involving the development of blood vessels around tumors implanted in rabbits' irises. Injections of fluorescein indicate that these new vessels are leakier than normal, and it was desired to synthesize fluorescently labeled macromolecules to quantitate the leakiness. Accordingly, we attached fluorescein isothiocyanate to dextrans of various molecule weights from 50,000 to 2,000,000. However, the fluorescence slit lamp apparatus combined with the relatively low degree of labeling has so far prevented observation of the injected fluorescent dextrans. Strategies to be pursued include heavier labeling, and Dr. Gullino is collaborating with BEIB to enhance the sensitivity of his apparatus.

3. Dr. Donald Fry, Chief of the Experimental Atherosclerosis branch of NHLBI, has been testing the effect of various diets on thyroidectomized dogs, which are susceptible to the development of atherosclerosis. With Dr. Robert Pitas of Meloy Laboratories, Springfield, Va., a contractor, Dr. Fry has noted that the various diets alter the lipid composition of red cell ghosts. In order to quantitate changes in membrane fluidity, Dr. Pitas has been collaborating using DPH as a probe and fluorescence polarizations performed with our laboratory. Results so far show an acceptable standard deviation, and the viscosities measured are consistent with reported data. Results so far suggest that the feeding of tallow and cholesterol do slightly increase the rigidity of red cell membranes.

Significance to Biomedical Research and the Program of the Institute:

These results contribute to the understanding of the nature of various fluorescent probes, which in turn provide information on the nature of binding sites on proteins or cell membranes. Fluorescence techniques are often the only convenient method for determining location, polarity, viscosity, and rotational mobility of such sites. The project therefore

is consistent with the development of techniques useful in biomedical studies, and we have applied this to problems involving lipids and red cell membranes of animals involved in experimental atherosclerosis.

Proposed Course:

We are continuing to study the fluidity of red cell membranes of amimals fed different types of lipids. The study on DPH is nearing completion and will exemplify the use of nanosecond polarization methods using the single photon method for determining heterogeneity of fluorescence decay. We also hope to obtain phosphorescence spectra in aqueous snows of proteins in their denatured state. This will be basic work in the field permitting us to identify those aspects of protein phosphorescence which are due to interactions with neighboring groups, and those aspects which are still present in completely unfolded protein chains. As other worthwhile opportunities for collaboration appear, these will be adopted as well.

Publications:

Abstract: Chen, R. F.: Fluorescence of Auramine O Bound to Serum Albumins and Yeast Alcohol Dehydrogenase. Federation Proc. 35, Abstract # 54, 1976.

Chen, R. F.: Fluorescence of Free and Protein Bound Auramine O. Arch. Biochem. Biophys. 179: 672-681, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01408-12 LTD

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Methodology in Fluorescence Measurments

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

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Technical Development

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INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.4	0.4

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The purpose of this project is to develop methodology for the use of luminescence methods in biomedical research. Major emphasis has been placed on the fluorescence quenching release method for assaying enzymes. In this method, a concentrated solution of a dye is encapsulated in a lipid membrane liposome made up of an amphiphilic compound such as phosphatidylcholine (lecithin). When attached by phospholipases, these liposomes release the dye into solution. The dye is nonfluorescent due to self quenching in the concentrated solution, but when freed into solution, the fluorescence quenching is released. This forms a basis for the assay of phospholipases, but could also be applied to a host of other liposome forming compounds and the enzymes specific for them. Another area of concern is the development of apparatus for orientation of fluorescent macromolecules. We have worked with modification of flow dichroism apparatus for this purpose.

PHS-6040

Objectives:

The long range objective is to develop methodology which will enhance the usefulness of luminescence spectroscopy.

Methods:

When instrumentation is being developed, we usually start with a commercially available instrument and modify it, using shop facilities, to enable it to be used for novel measurements. When developing new methods in fluorescence, we make use of presently available spectrofluorometers, lifetime devices, etc. for the assessment of quantum yields, lifetimes, and other relevant parameters.

Major Findings:

1. A novel fluorimetric method, which we call fluorescence quenching release (FQR) has been developed. When dispersions of lecithin (phosphatidylcholine) are sonicated in 0.2 M carboxyfluorescein, liposomes having bilayer membranes are formed which are virtually nonfluorescent because the enclosed dye is concentration-quenched. When the liposomes are disrupted by detergents, the dye is released, and there is usually a 20-100 fold increase in fluorescence. We have prepared liposomes from various types of lecithin, with or without cholesterol, which stabilizes the liposome membranes, and used these dye-containing liposomes as substrates for the assay of phospholipases. These enzymes hydrolyze various parts of the lecithin molecule. The usual assay of the enzymes requires titration of the fatty acids released from lecithin, a time-consuming method. When the FQR method is used, the enzyme activity is proportional to the fluorescence which is developed. This optical method lends itself to rapid, sensitive assay of multiple samples, and to automation. The method has been tried with commercial preparations of phospholipase A from snake venoms, bee venom, and phospholipase C from C. welchii and B. cereus. Although these enzymes hydrolyze different parts of the lecithin molecule, sufficient disruption of the liposome structure occurs that dye is released and the enzymes can be assayed by FQR. FQR could be applied to the determination of small amounts of detergent, which also disrupt the liposomes. FQR may also be useful to assay other enzymes which attack other liposome-formers, such as sphingomyelinase. In addition to the great promise of FQR for analytical biochemistry, FQR also allows optical studies of the kinetics and mechanism of many of these enzymes of lipid degradation and therefore will probably contribute to basic enzymology.

2. Some experiments have been carried out with Dr. Y. Ito of this laboratory on the separation of dye-labeled protein conjugates. These proteins, when reacted with dyes for the purpose of making them fluorescent for either immunochemical or biochemical studies, probably form a very heterogeneous

population. When the preparation contains an average of N molecules of dye per molecule of protein, there are probably protein molecules present which contain from 0 to N + i dyes. The theory of fluorescence depolarization used to determine the relaxation time of proteins assumes random labeling. To test the random labeling hypothesis, we have prepared dye-protein samples for Dr. Ito to separate on his polymeric 2-phase system of countercurrent chromatography, and he has been able to separate these samples into different fractions with different dye/protein ratios. Discrepancies have been noted with the random labeling assumption, although the separations have not been as ideal as desired as yet. Another advantage of separating these species of dye-protein molecules is to have preparations which are homogeneous; this would be of use in energy transfer and polarization studies.

3. A Shimadzu UV-50 flow dichroism device has been obtained from Dr. Martin Gellert of NIAMDD, with the aim of modifying it to permit fluorescence measurements of oriented macromolecules. The device will require front face illumination and detection of the fluorescence, and attempts to incorporate the device into the Aminco-Bowman spectrofluorometer have so far been unsuccessful. Other means for orienting molecules, as well as other modifications to the Shimadzu device are being eyed for this project. The orientation of samples would be of use in determining the direction of absorption and emission dipoles, and for the measurement of relative orientation of dyes bound to elongated macromolecules such as fibrinogen or DNA.

Significance to Biomedical Research and the Program of the Institute:

The first spectrofluorometer was developed in this institute some 20 years ago, and the laboratory has continued to contribute to the development of fluorescence technology. The technique of fluorescence spectroscopy is already most useful in biomedical studies, but its full potentialities have by no means been attained.

Proposed Course:

The fluorescence quenching release study for enzyme assay will be continued and written up. A different report could be prepared for each enzyme assayable by this method, since some differences in technique might be required in each case. The extension of the method and various refinements could be most fruitful. Specifically, one could investigate different kinds of liposome membranes, the conditions and additions required for optimum stability and low background, the different enzymes which could be assayed. Non-enzyme assays, such as the assay of detergents and other substances which could disrupt the liposomes are conceivable. Where traditional enzyme assays require cumbersome titrations, we intend to show that FQR will be useful for rapid kinetic studies and studies of enzyme activation and inhibition by compounds such as fatty acids which would interfere with

titrations.

The problem of orienting samples for fluorescence polarization measurements will be approached by 1. use of capillary flow ensembles developed by Drs. Bowman and Moscowitz and 2. use of the Shimadzu UV-50 flow dichroism device with front surface illumination.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01409-06 LTD

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Develop Instrumentation for Assaying Mammalian Cell Parameters

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

PI: P. Carmeci Electronic Engineer LTD NHLBI

COOPERATING UNITS (if any)
Hematology Service, Clinical Center, NIH
U. S. Naval Hospital, Bethesda, Maryland

LAB/BRANCH
Laboratory of Technical Development

SECTION

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NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Using capillary flow techniques a new method for measuring the fragility of red blood cells has been developed. Efforts have been expended in making this method more highly automated and also in demonstrating the advantages of this new instrument for identifying other cell characteristics and parameters.

435

Objectives

The objectives of the project are:

1. To evaluate capillary flow techniques for measuring red blood cell fragility.

2. Demonstrating this technique for measuring other types of cells.

A. Measurement of Red Blood Cell Fragility.

A simple and accurate method for the determination of cell osmotic fragility has been investigated. The method uses laminar parabolic flow pattern together with gravity to retain cells in a long capillary tube while a solution with decreasing osmolarity is passed thru the tube for hemolysis. Hemoglobin released from the cells is quickly removed by the axial flow pattern and monitored with a spectrophotometer for recording the hemolysis curve. To demonstrate the capability and advantages of this technique comparisons were made of groups of normals using present methods and this new technique. In addition, a group of infants were also tested and "norms" established. Also a number of patients with RBC dysfunctions such as thallasemia, spherocytosis and sickle cell anemia were observed with this technique.

Using this same technique, a means of testing a number of sample simultaneously is being investigated. Efforts have been expended in designing and building a device that will Test 8 samples at a time, and be relatively simple in design and operation. It consists of a pre-programmed tape recorder and air valve to provide the NaCl gradient, a pump to provide the flow and a manifold with 8 sample ports and capillary tubing. The monitoring device will measure both salt concentration and hemoglobin in the same cell thereby eliminating calibration of the device.

Investigations are being made to utilize this technique as a research tool for measuring other parameters. The instrument has been modified so that the fragility of sickle cells can be assayed under oxygenated and deoxygenated conditions. The instrument has also been modified to assay the lysis of RBC with a gradient of antibody complement. Also demonstrated was the study of the effect of high cholesterol upon RBC fragility.

Significance to Biomedical Research

The measurement of RBC fragility offers the advantages of:

1. small sample size

2. output in a non-integrated form

3. no sample preparation necessary

4. accuracy and reproducability is higher

5. the method is amenable to changing parameters and studying other types of cells.

Proposed Course

A. Continue with modifying the method so that a number of samples can be assayed simultaneously and to demonstrate the applicaility of this technique to other parameters and other types of cells.

B. Due to the increased use of hyperthermia as a theraputic agent and as an adjunct to chemotherapy and radiation, it is felt that more sensitive assays can be performed on biopsy and blood specimans by culturing specimans in a temperature gradient rather than the present methods used. Efforts will be expended in devising a thermal gradient cell culturing system for a specific purpose.

Publication:

Carmeci, P., Ito, Y. and Steele, R.: Continuous flow method for determination of eythrocyte osomotic fragility. American Journal of Hematology. 2-4: 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01410-03 LTD

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Blood Gas Monitoring for Extended Periods

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

PI :	G. Vurek	Senior Investigator	LTD NHLBI
OTHERS:	T. Kolobow	Chief, Section on Pulmonary and Cardiac Assist Devices	LTD NHLBI
	T. Clem	Electrical Engineer	BEIB 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 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	3/4	1/4

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

This project involves the development of a system to measure blood PO_2 and PCO_2 in extracorporeal circuits for 72 hour periods without blood loss. Picomole amounts of oxygen and carbon dioxide are extracted from the circulation with a special flow through probe or catheter probe. Oxygen is analyzed electrometically and carbon dioxide is measured by calorimetry of the reaction with carbon dioxide and lithium hydroxide. Tests have shown the system to be stable within five percent over 72 hour periods.

Objectives:

The objective of this project is to make a system which will provide continuous information about blood PO_2 and PCO_2 during extracorporeal procedures with membrane lungs. The system does not require removal of blood from the circuit and can operate without calibration more frequently than once per day. The system will provide these measurements during an extended extracorporeal circulation procedure.

Methods Employed:

There are three parts to the system: The sampling probe, the analytical instrument, and the data handling circuits. We avoid the use of conventional Clark-type oxygen electrode and Severinghaus-type PCO_2 electrodes because these electrodes must be calibrated frequently in order to maintain good precision and accuracy. Therefore, we are limited to extracting gases from the blood by diffusion which requires sensitive transducers. Gases diffuse from the blood through a permeable membrane into a stream of purified nitrogen which then sweeps the sample gases to the analytical system. The gases enter the stream of nitrogen carrier gas in proportion to their partial pressures within the blood. Because of the complex relation between O_2 content, CO_2 content, and their respective partial pressures it is important to measure the temperature of the blood at the sampling site. This permits us to correct the probe response for changing blood temperature and to account for the difference between our measurement and measurement on drawn samples using conventional blood-gas instruments.

The carrier gas with the O_2 and CO_2 from the sample arrives at the analytical instrument where it passes to a two way valve. Approximately every 65 seconds the valve diverts the gas from the main stream into the carbon dioxide analyzer. This diversion occurs for 5 seconds. The rest of the time the gas either passes to the oxygen analyzer or to the outside air. Approximately 12 microliters per second of carrier gas is consumed by the probe system. Carbon dioxide in the carrier gas reacts at the surface of the solid porous granule of lithium hydroxide intimately contacting a thermistor bead. The heat of reaction, approximately 9×10^4 joules per mole, causes the temperature of the bead to rise and this temperature rise produces a signal which is integrated electronically. Oxygen has been measured using a commercial oxygen analyzer which converts the gas to hydroxl ions by reducing each molecule with four electrons. Approximately three hundred picomoles of carbon dioxide are consumed at normal partial pressures for each 65 second period and approximately 10 picomoles per second of oxygen arrives at the oxygen analyzer producing a current of 26 microamperes. The noise level of the system is such that measurements at normal partial pressures of oxygen and carbon dioxide can be made with a precision of $\pm$ 3%. The electrical signals from the sampling

probe thermistor, the carbon dioxide calorimeter and the oxygen sensor are converted electrically with the aid of a microprocessor. A microprocessor based read-out unit converts the transducer outputs to digital numbers corrected to 37°C. The system, developed in close collaboration with the staff of the Biomedical Engineering and Instrumentation Branch, DRS, is described more fully in their project, Number Z01 RS 10025-01. This blood gas analyzing system can provide current and average data on PO_2 and PCO_2 as well as display the output of blood oximeter that has previously been developed in this laboratory.

Major Findings:

A satisfactory flow-though probe design has been achieved and we have started to explore a design for a small catheter tip probe. The flow-through probe consists of a molded piece of silicone rubber 9 mm inside diameter with a side port approximately 15 mm across. The side port accepts the sampling system which is a piece of polyethylene glued to a brass cylinder with silicone adhesive. The entire inside of the probe is coated with silicone gum according to the technique developed by Dr. Kolobow. This inner silicone coating adheres tenaciously to the silicone rubber and the polyethylene when it has been treated with an oxidizing acid. Approximately 0.5 cm^2 of polyethylene is available for diffusive gas transfer from the blood to the carrier gas stream. The probe can be sterilized with ethylene oxide or other cold sterilization procedures. It is separable from the tubing which carries the gas to and from the analytical instrument. The two parts are joined with a screw cap and sealed with an o-ring. Although the probe does not have the describable features of the completely unit-molded probe tried earlier, it is easy to fabricate and the separability feature makes it much more convenient to handle when inserting into the blood circuit.

In addition to the flow-through probe we have some initial trials of a catheter-type probe. This probe is a dual-lumen very flexible catheter approximately 0.5 mm outside diameter. The catheter is made of stainless steel and Kel-F tubing, polypropylene tubing, and Teflon. A piece of 50 μm wall Teflon tubing is epoxyed over the end of the Kel-F; its tip is plugged with epoxy. Carrier gas passes down the inside polypropylene tube and sweeps gases which diffuse through the permeable Teflon back up the Kel-F and stainless steel tubes to the analytical instruments. This probe was developed because it seemed an appropriate assessory to the blood gas analyzing system. The sensitive part of the probe consists of the three centimeter length of Teflon tubing. This provides sufficient permeability for the proper operation of the analytical instruments. There is no data available at the present time on the response time or influence of the passage of blood past the probe.

A major fraction of this year's work has been devoted to making the carbon dioxide system more reliable. We have previously reported that it performs satisfactory for periods exceeding twenty four hours. But this was not always the case and quite frequently very erratic results have been obtained. As previously described, the reaction of carbon dioxide and lithium hydroxide involves the simultaneous release of three moles of water per mole of carbon dioxide absorbed. It is the subsequent reabsorption of most of this water which produces the perceptable heat output of the reaction. Therefore, it is absolutely necessary to maintain the lithium hydroxide in the proper state of hydration. This has been accomplished by controlling the humidity of the carrier gas with a lithium hydroxide hydrater. This device contains approximately one gram of fully hydrated lithium hydroxide which releases water as the dry nitrogen carrier gas passes over it. The partial pressure of the water is exactly that which will maintain the lithium hydroxide at the reaction site in the necessary degrees of hydration. In addition the lithium hydroxide used in the analytical segment must be stored over lithium hydroxide to maintain the proper storage humidity. We observed that properly prepared granules and humidified carrier gases can provide continuous measurements for periods exceeding three weeks, which approaches the theoretical carbon dioxide absorbing capacity of a single granule. During these tests, the probe and analytical system were periodically tested for linearity and base-line stability. The linearity of the standard curves always showed correlation coefficients greater than .99 and the baseline stayed within 2 Torr; sensitivity shifted less than $\pm$ 10% during the test. Although there is still some erratic behavior when granules are changed, in general we find that the system recovers fom exchanging lithium hydroxide granules within 15 minutes and fully recovers its sensitivity within one hour. If a fresh granule does not show satisfactory return to baseline within 15 minutes that is sufficient evidence to reject that granule's performance and apply a new one. Granules can be interchanged quite easily without disrupting the blood system.

The oxygen analyzing portion of this system has not been satisfactory. We have used a commercial coulometric oxygen analyzer which performed satisfactory initially, although it's response time was rather slow. This was acceptable because the periodic sampling needed for measurement of carbon dioxide placed a lower limit on the useful oxygen sensor response speed. During long use of this system it became apparent that the commercial analyzer had deteriorated and developed a response time exceeding two hours. The sensor continued to show response to changes in oxygen but the final 10% response to step changes required this two hour period and we decided this performance would be unexceptable for a dynamic measurement system. We are therefore exploring alternative oxygen sensors including one based on the mesh electrode described in project Z01 HL 01418-02. In this electrode oxygen passes through a silver mesh from the carrier gas stream into a electrolyte solution. The mesh is immediately adjacent to the entry point of the oxygen into the electrolyte

and therefore the dissolved oxygen reacts at the silver to produce an electrical current. Our present activity involves preventing the electrolyte from penetrating the pores of the mesh and obstructing them, and reducing the background to zero current.

We have made several in vivo tests of the entire system in collaboration with Dr. Kolobow's group. Although the experimental conditions of the procedures being performed by Dr, Kolobow's group prevented frequent comparisions of actual blood gas measurements with displayed PCO_2, the apparatus performed satisfactory and gave reliable and useful reading during the procedure.

Significance to Biomedical Research and the Program of the Institute;

This instrument system will permit measurement of blood gases in experimental animals or patients. Because it does not require removing blood samples from the circuit nor frequent calibration, it can provide these data safely and continuously during prolonged procedures. It also demonstrates the feasibility of alternate methods for blood gas analysis other than conventional electrodes, gas chromatography, or mass spectrometry.

Proposed Course:

The carbon dioxide measuring part of the system and flow through probes will be written up for publication. In addition, further work on a reliable and satisfactory oxygen sensor will go forward so that the entire blood gas analyzer system project can be completed.

Publications: None

PROJECT NUMBER (Do NOT use this space)	HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01411-11 LTD

PERIOD COVERED

July 1, 1976 to September 30, 1977

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 Technician	LTD NHLBI
	R. L. Bowman	Chief, LTD	LTD NHLBI
OTHERS:	A. Sances	Professor and Chairman Biomedical Eng.	Medical College of Wisconsin
	J. H. Battocletti	Research Associate	Medical College of Wisconsin

COOPERATING UNITS (if any)

Medical College of Wisconsin, Milwaukee, Wisconsin

LAB/BRANCH

Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	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)

Using the idea that nuclear magnetism of protons in water persist for the greater part of a second, we are developing methods for tracing and measuring blood flow that work entirely thru electromagnetic fields. Protons in water of the blood polarized by a strong static magnetic field retain the polarization and thereby produce a stronger signal in the downstream blood than umpolarized blood. In the tracer mode an oscillating field is used to erase the polarization at a specific point to discriminate the proportion of blood that passed through the erasing field.

Various locations of the polarizing and erasing field are used to provide information about blood flow in various organs. A specific aim is to provide an instrumental basis for measuring intracranial blood flow and distribution to facillitate studies of intracranial atherosclerosis and stroke.

PHS-6040
(Rev. 10-76)

Project Description

The project consists of an electronics section devoted to the development and refinement of special circuits and modes of operation and a contract facility concerned with the testing and evaluation of the apparatus in experimental system using animals and models in preparation for clinical application.

In this laboratory, we are developing special circuits for specific applications to assess the sensitivity and specifity for particular medical applications.

In order to evaluate certain problems of tissue water interference and the sensitivity of the method we built a system that could be tested on finger blood flow. The digital flow problem was also considered of interest as a peripheral vascular research tool. The finger would provide a small flow system that could be tested easier than cerebral flow.

One of the problems of measuring low flows is the fact that a small vessel is surrounded by a large volume of tissue containing protons that are detected along with those protons in the flowing blood. Discrimination in favor of flowing protons and against stationary protons was provided by working at a relativly low detector field and prepolarizing the blood before it entered the detector field thus the static protons produced almost no signal in the weak field while the strongly prepolarized protons entering the detector by flow produced relativly strong signals. Additional discrimination is produced by modulating the erasure coil which modulates only that signal from protons that have traversed the erasure volume.

The system was assembled in model form using a 0.5 mm vessel for flow surrounded by a larger tube containing about 10cc water, (static protons) at a flow of a few cubic milimeters per min. a signal to noise ratio of 3 was obtained indicating that this system had sufficient sensitivity to measure clinically significant changes in digital blood flow.

The evaluation was obtained on a makeshift arrangement that was not suitable for definite experiments on the finger so that is was decided that the total system consisting of NMR reciever and transmitter should be converted to the new crossed coil mode used in finger experiments.

The electronic system in use up to this time demonstrated that several modes of operation could be used to detect and trace flows.

To make the best use of the selected systems the electronics were redesigned and reconstructed using state-of-the-art technology for

obtaining the optimal performances for use with the new high field superconducting magnet to be installed at our contract facility at M.C. of Wis. The new electronics is completely ready for 100 KHz detection at 2.35×10^3T, 7.15 MHz@ 0.12T, 9MHz@ 0.2T and 75 MHz at 1.8T already complete and will permit use of the each of several modes of operation at Wisconsin.

At the Medical College of Wisconsin during this contract year, tag detect methods were used to retrieve cerebral blood flow signals from the confluence of the sinuses at the region of the jugular bulb. Since the blood is demagnetized atothe confluence of the sinuses, the amplitude of the retrieved signal is proportional to flow rate. The transport time from the demagnetized area to the detection region can also be determined. A method has been derived for the calculation of the area of the vessel and the volumetric flow rate.

Additional electronic circuitry, mechanical and magnetic improvements made in the 12.5 cm diameter cylindrical, crossed-coil limb flowmeter now make it possible to record arterial blood flow without averaging. The NMR flowmeter has detected substantial differences between flow in the normal arm and one with subclavian obstruction.

A flat crossed-coil detector was developed for application to the surface of the body. A model arm unit with a brachial artery, cephalic vein, basilic vein, median artery and vein, ulnar artery and vein constructed. With this system, the cylinderical crossed-coil NMR flowmeter was quantitatively compared with the flat crossed-coil system. Arterial signals could be retrieved with the same signal and amplitude independent of position within a 5 cm radius. Similar findings were observed with the venous signals using tag-detect methods. Similar sensitivities for the flat crossed-coil in comparison to the cylindrical crossed-coil system were obtained in the human arm. The flat crossed-coil system is consequently the system of choice for future surface recording systems. When the cylinder was filled with doped saline to simulate surrounding tissue, some additional noise was introduced but not enough to significantly interfere with the signal retrieval for flows as low as 50 ml/min.

The design of the 9MHz flat crossed-coil electronic system is nearly complete. The receiver transmitter and power module have been completed. Because of the need to explore the increase in sensitivity with higher magnetic field systems, a 63.5 cm internal diameter, 185 cm long cylindrical magnet has been ordered. This system will be capable of developing 2T within the lumen. Calculations predict an 80 times sensitivity increase at 2T over the 0.2T system (for a time delay of 2.5 seconds). The magnet is scheduled for delivery in June 1977.

Objectives:

To develop non-invasive methods of measuring and tracing blood flow using nuclear magnetic resonance technology.

Methods Employed:

Electronics and physical techniques are used to provide field and frequencies of electromagnetic radiation that sense the susceptible protons contained in the blood.

Significance to Biomedical Research and the Program of the Institute:

NMR methods have great potential for tracing and measuring either protons or other susceptible nuclei either normally present or introduced. The non-invasive potential with the harmless nature of the radiation makes the concept partially attractive for chronic observation or frequent use without morbidity. A flow system that could follow vascular changes intercerebrally without harm has been identified as a major problem in cardiovascular research for pathology leading to stroke.

Proposed Course:

1. Continued development of electronic methods of NMR measurement.

2. Application of the apparatus developed here to animal experiments at the Medical College of Wisconsin using the superconducting magnet.

3. Development of a practical clinical system for evaluating regional cerebral flow by a completely harmless, non-invasive method.

Publications:

1. Sances, A., Jr., Battocletti, J. H., Halbach, R. E., Larson, S. J., Evans, S. M., Bowman, R. L., and Kudravcev V.: "Limb blood flow measurement by NMR", Digests of XI Int'l Conf. on Medicine and Biology in Engineering, Ottawa, Canda, August 1976, pp. 430-431.

2. Malfertheiner, P., Sances, A., Jr., Battocletti, J. H., Larson, S. J., Halbach, R. E., Evans, S. M., Bowman, R. L., Fegiz, G., and Ray, L.: "NMR (nuclear magnetic resonance) a non-intrusive technique for measurement of blood flow", Surgery in Italy 6(3): 186-194, September 1976.

3. Halbach, R. E., Battocletti, J. H., Sances, A., Jr., and Evans, S. M.: "Limb blood flow monitoring by a NMR probe", Clinical

Research 24:559A, Oct. 1976.

4. Battocletti, J. H., Halbach, R. E., Sances, A., Jr., Larson, S. J., Bowman, R. L., and Kudravcev, V., "Cerebral blood flow measurement using nuclear magnetic resonance techniques", Proc. 29th Ann. Conf. on Engineering in Medicine and Biology, Boston, Mass., 1976, p.42.

5. Battocletti, J. H., Halbach, R. E., Sances, A., Jr., Larson, S. J., Bowman, R. L., and Kudravcev, V.: A flat crossed-coil detector for blood flow measurement using NMR, submitted to 30th Ann. Conf. on Engineering in Medicine and Biology, Los Angeles, Cal., 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01413-15 LTD

PERIOD COVERED
July 1, 1976 to September 30, 1977

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, Section on Biophysical Inst,	LTD NHLBI
	P. Smith	Expert Consultant	LTD NHLBI
OTHERS:	J. Froehlich		Nat. Ageing Inst,
	F. Kregenow		KE NHLBI
	R. Podolsky		PB NIADD

COOPERATING UNITS (if any)
Thermometrics, Inc., Dover, New Jersey
Biomedical Engineering and Instrumentation Branch, NIH

LAB/BRANCH
Laboratory of Technical Development

SE
Section on Biophysical Instrumentation

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) A stopped flow microcalorimeter capable of measuring first order reaction rates of 5 x 10^{3} sec^{-1} and temperature changes of 0.0005°C has been developed. This calorimeter is adiabatic to 2% for 2 sec., returns to equilibrium in less than 5 minutes and uses 100 ul of each solution per reaction. It utilizes a thermistor with 3 msec rise time. A mixer has been developed that gives a temperature rise of less than 2 x10^{-3} C due to mixing heat at a flow velocity of 2 m/sec. Computer programs using the finite element technique to simulate the thermal properties of the calorimeter, correct for heat losses, and finite thermistor response time have been developed. A liquid dye laser flash photolysis system producing a 1 microsecond duration output has been utilized in an instrument for the study of oxygen and carbon monoxide binding to human hemoglobin. Interpretation of the observed kinetics in terms of a two state Monod Wyman, and Changeux type model has been analyzed and simulated on the Decsystem 10 using a finite element simulation approach. Equilibrium as well as kinetic information is obtained. Extension of the detector system for use with red cell suspensions is under way utilizing dual wavelengths at 660 nm and 900 nm. This instrument has also been modified to allow the use of combined stopped flow-flash photolysis.

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Project Description

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 the method and instruments to study, in collaboration with other laboratories, the reactions of hemoglobin with the respiratory gases both in the normal state and as modified by the changes of physical factors, small molecules, various metabolites, and genetically, such as in sickle cell anemias. The reactions of various cellular enzymes, particularly ATPase and lactate dehydrogenase, and their interactions, and control, in the cell are studied as they relate to the hemoglobin reactions in cardiology, pulmonary, and respiratory function, and curculation. 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 the several special research laboratories such as the Jet Populsion 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 applied to certain specific enzyme systems under investigation.

Results:

The investigation of the reaction CO_2 and DPG with hemoglobin has resulted in the development of a new stopped-flow microcalorimeter. The instrument has the following attributes:

a. adiabatic to 2% for 2 seconds

b. returns to equilibrium temperature in less than 5 minutes.

c. uses 100 u liters of solution or less per reaction.

d. is capable of following first order reaction rates of 5×10^3 sec^{-1}.

e. is capable of temperature measurements on the order of $0.0005^{o}C$.

Calorimetry is a technique of general applicability because most chemical processes of biological interest involve energy changes. Unfortunately there is a technical problem of measurement because most other processes such as flow through tubes mixing of solution and pressure pulses also cause temperature changes and these are basic to the design of a stopped flow calorimeter.

A major problem is the study of the heat generated in mixing and the stability of the flow patterns in the observation tube during acceleration, constant flow velocity and stopping. Studies of this problem have been made in this past year. An electro-magnetic flow meter was used to study the flow patterns, a piezo-electric pressure transducer to observe the pressure drops in the system and thus test the hydrodyamic efficiency. A fast thermistor (3 msec rise time) was used to follow the heat generated during flow in the tubes and mixer and to study the temperature gradients and equilibrium times.

Calculations to determine the amount of heat generated in the total mixer have been carried out and compared with the experimental results. Finite element simulation of the calorimeter and thermistor thermal response has been carried out to allow data correction for both thermal and time response. Excellent agreement has been achieved so that a much clearer understanding of the optimum design was obtained. Several different experimental arrangements were investigated as the system was developed.

The Rodamine 6G liquid dye laser produces a 1 Joule, 1 microsecond output at 580 nm. This pulse overlaps the α-absorption peak of oxyhemoglobin, and is thus suitable for the photolysis of oxygen from oxyhemoglobin. An instrument comprising the laser together with a sample compartment has been constructed and tested. 80% photolysis of 100uM hemoglobin samples has been readily achieved. The sample cell is monitored by a tungsten iodide lamp source; transient kinetics produced by the laser pulse are recorded using an interference filter photomultiplier combination and the results recorded directly on a Biomation a/d converter.

Modification to the Datos-Data Graphics interface now allows remote usage of the system and presents the data in a more amenable form. Computer programs have been written to separate the data into individual runs, and conversion from transmission to absorbance changes.

Analysis of the hemoglobin data has been treated by the finite element analysis simulation technique. A kinetic model of hemoglobin function

based on a two state MWC type model has been simulated on the Decsystem 10 using the finite element technique for the various cases of ligand binding to hemoglobin; for example, presence or absence of phosphates and 2,3-DPG. The simulation technique is being extended to account for α, β differences in ligand binding, thus making the model suitable for treating oxygen binding to hemoglobin. From the kinetic data obtained experimentally using the laser photolysis apparatus, predictions of the equilibrium behavior can be obtained generating the oxygen dissociation curve.

A detector system suitable for work with whole blood and red cell suspensions has been constructed and is in the process of being calibrated. Two light emitting diodes of 660nm and 900nm chopped at 1Mhz are employed, and alternately pass through the sample. The two beams are separated using dichroic mirrors in combination with interference rejection filters. The resulting beams are detected using a pair of photo transistors and the output fed to a differential log amplifier. The absorbance induced by the laser photolysis pulse, are all available as outputs from the detection system. The system currently operates at 1 MHz but it is expected to be able to operate at a faster rate once it has been tested.

The study of the binding of calcium to EGTA has been continued. Experiments were performed on the high speed optical stopped flow apparatus at concentrations ranging from 1mM to 0.1 mM of each reactant at both 25°C and 7°C. The work was performed in cacodylate buffer at pH 7.0 and the reaction observed by monitoring the absorbance change of bromthymol blue in response to the hydrogen ion released in the binding process. Analysis of the resulting data was treated by the finite element technique and verified by separate analysis using the Mlab facility on the Decsystem 10. Rate constants of 3×10^6 $M^{-1}S^{-1}$ at 25°C and $7.10^5 M^{-1}S^{-1}$ at 8°C were obtained.

Proposed Course:

Calibration and reactions with red cells will be continued in collaboration with Clinical Hematology. Ca-EGTA work will be continued to clear up the reaction mechanism.

Work on a new rapid filtration apparatus has been started as a modification to quench flow system. The movement of Cl^{31} out of the duck red cell is the biological system being studied. Preliminary results indicate that roughness of the filtration membrane contributes greatly to hemolysis so that nucleopore filters will be tried in the future. Good results were obtained on several experiments indicating outward movement slower than 50 milliseconds by a considerable amount at 27°C.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01414-05 LTD

PERIOD COVERED
July 1, 1976 to September 30 1977

TITLE OF PROJECT (80 characters or less)
Development of Microcalorimeters for Clinical Chemistry

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

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	M. Marini	Consulting Biochemist	N.W. Univ. Med. Sch.
	N. Davids	Consulting Mathematician	Penn. State Univ.
	J. Everse	Consulting Biochemist	Tex. Tech. Med. Sch.
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COOPERATING UNITS (if any)
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Clinical Chemistry Department, Clinical Center, NIH

LAB/BRANCH
Laboratory of Technical Development

SECTION
Section on Biophysical Instrumentation

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	3	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A fast thermoelectric equilibrator has been constructed for use with the stopped-flow calorimeter and found to bring the solution to temperature equilibrium to less than 50 μ calories when 150 μ liters of each reagent are injected. A bubble inserter to separate samples has been developed and is undergoing tests. A new differential thermal-pH titration cell has been constructed and is undergoing tests. Titration of a protein from pH 5 to 10 can be accomplished in 2 minutes. Mlab programs give pk's and heats of ionization which agree, within experimental errors, of model compounds with other methods.

Project Description:

Objectives:

Virtually all chemical reactions produce heat and calorimetry has long been used to investigate them. For biological use, however, high sensitivity, small volumes of reactants, and short equilibrium times are needed. It is the objective of this project to develop such an instrument for use in the time range of a few seconds to 1 or 2 hours.

Method 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 calorimitrists utilizing appropriate enzymatic and cellular reactions.

Major Findings:

The microcalorimeters operating in Clinical Chemistry have been used for the determination of a variety of enzyme substrate-reactions, including the protolytic activity in fecal matter, cholesterol, triglycerides, and uric acid in serum. Details will be given in the Clinical Chemistry Report. Work continues in this laboratory on improving the performance of the present instrument by improving sample cells, some improvement in the amplifier electronics and thermal regulators, and introducing a microprocessor to take and correct the data so that immediate on-line results can be obtained. Microprocessors will also be used for totally automating the flow calorimeter. A fluidic logic flow system has been constructed which permits the injection of a buffer enzyme in a carefully controlled manner so as to minimize the mixing artifacts to less than 1 microcalorie. Work has been completed to bring the thermal equilibrium of the entering reactants to the same low level. A Glucose Thermistor sensor has been constructed and tested with glass beads glued to the thermistor bead. Results look encouraging.

Proposed Future Research:

The effectiveness of the batch microcalorimeter as an insturment suitable for routine clinical work and as a method of absolute standardization of biochemical work will continue to be explored in collaboration with Clinical Chemistry and Clinical Hematology. Additional exploration of other chemical reactions is planned, particularly in the area of antigen-antibody reactions as well as the determination of 2,3 DPG in the red

cell. Considerable work is needed to solve a number of technical problems associated with high reliability and sensitivity of the stopped-flow microcalorimeter and these will continue to be pursued. Development of the enzyme thermistor sensor will be continued, using glucose oxidase from P. Notatum, and efforts are underway to immobilize the enzymes directly on the thermistor bead.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01415-04 LTD

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Italy-U.S. Cooperative Science Program-Blood Gas Instruments-Projects 78

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

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	R. Winslow	Clinician	CHB, NHLBI
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	C. Gibson	Electrics Eng.	BEIB DRS
	L. Thiabault	Mechanical Eng.	BEIB DRS

COOPERATING UNITS (if any)

Biomedical Engineering and Instrumentation Branch, DRS, NIH

LAB/BRANCH

Laboratory of Technical Development

SECTION

Section on Biophysical Instrumentation

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	1.5	1.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The Italian-U.S. Science Cooperative project has thoroughly tested the oxygen dissociation curve apparatus designed for both clinical and research use. A large number of normal and abnormal hemoglobin patients have been tested. Complete automation of the system has been achieved through the use of a microprocessor.

PHS-6040

Project Description

Objectives:

The total oxygen needed by a normal subject is provided by the circulatory system according to the well known equation:
O_2 consumption = cardiac output x arterial-venous O_2 difference.
Since several pathological conditions can shift the oxygen dissociation curve (ODC) and thus how much oxygen can be released to the tissues, it is of considerable clinical and fundamental physiochemical interest to be able to measure the ODC under true physio logical conditions of the patient. The aim of this project is to develop instrumentation to provide a comprehensive analysis of the various chemical factors regulating the (A-V) O_2 difference or, more generally, the oxygen dissociation curve of human blood under various physiological or pathological conditions. ODC position and shape is under control of various small molecules or ions, i.e. CO_2, protons, and 2,3-DPG, etc.

Methods Employed:

A systematic analysis of the complex interrelationship among several variables and their effect on the oxygen dissociation curve requires the development of a simple 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 in the clinical Hematology Branch, NHLBI. 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. Kolobow.

Major Findings:

The oxygen dissociation curve apparatus has been installed in the Clinical Hematology Branch and is being used daily for patients.

A new automated system based on the Intel 8080 microprocessor has been constructed and put into operation. At least 15 OEC's are run per working day. Extensive file handling capabilities have been developed for the Tetronix Floppy Disc which has been interfaced to it.

A new system using an oxygenator membrane is under development. This would allow the curves to be run from oxy to deoxy and back in thirty minutes without the use of Hydrogen Peroxide or a tonometer.

This will be very useful in resolving the question of the hysterysis found in Sickle Cell Anemia. It will also allow OEC's to be run on concentrated Hb solutions. This is not possible with the present instrument since Hydrogen Peroxide interacts with the protein. A portable version will be constructed for field use.

Publication:

Winslow, R., Swenberg, M., Berger, R., Shrager, R., Luzzana, M., Samaja, M., and Rossi-Bernardi, L.: Oxygen equilibrium curve of normal human blood and its evaluation by adair's equation. The Journal of Biological Chemistry. 252: pp. 2331-2337, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01418-02 LTD

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Fast Responding Oxygen Electrode

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

PI :	G. Vurek	Senior Investigator	LTD NHLBI
OTHERS:	I. Levin	Special Expert	LTD NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20014

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)

This project is concerned with the design, construction and evaluation of an oxygen electrode with quantitative electrolytic reduction of oxygen, low oxygen consumption per unit area, and rapid response. We are studying the application of silver mesh as cathode material on the performance of the electrode system with the above properties. The low porosity silver mesh should reduce the oxygen consumption per unit area and avoid the usual problem of variable electrolyte thickness between cathode and protective membranes.

Objectives:

The purpose of this study is to develop an oxygen electrode with no electrolyte barrier between the protective membrane and the oxygen reduction cathode. In addition, the use of porous cathode material should reduce the oxygen consumed per unit area while at the same time maintaining a rapid response time.

Methods Employed:

Conventional Clark-type oxygen electrodes have used membrane made of polymers to separate the oxygen cathode from unwanted electrochemically active species in the sample. In addition to the diffusion barrier presented by the membrane there is usually a layer of electrolyte between the membrane and cathode. The thickness of this electrolyte must be stabilized in order to have precise response of the electrode to sample oxygen. The approach we have chosen to follow in this project consist of intimately bonding the cathode to the polymer membrane so that there is no electrolyte barrier between them. Oxygen reaches the electrolyte through holes in the cathode and reacts at the back surface. By using a low porosity cathode structure the resistance to oxygen can be made high without either increasing the diffusion path or without using a material with low permeability. Isotropic materials with the latter property also have long response times.

Major Findings:

The work of the current year has been devoted to finding an appropriate adhesive system and evaluating electrolyte compositions. The role of the adhesive is crucial because it is imperative to prevent a electrolyte from penetrating between the cathode and protective membrane. The adhesive must be highly permeable to oxygen so that it does not interfere with properties of the overall electrode and yet have good hydrolytic stability and resistance to alkali. The presence of a minute layer of electrolyte between membrane and cathode degrades the performance of the electrode. We have tried silicone adhesive with and without primers on silver cathodes and gold cathodes. None have survived more than a day of use. We are presently investigating a polyurethane heat setting film adhesive. Initial test seems promising. Electrolyte composition is also being tested because the reduction of oxygen at a cathode can proceed to water at low pH or hydroxyl ions at high pH. It seems desirable to avoid generating large quantities of alkali at the cathode. Unfortunately the low pH buffers we have tested to date have all produced excessive background currents, probably due to the reduction of hydrogen ions at the cathode in competition with oxygen reduction. We have confirmed that the oxygen electrode will respond to changes in oxygen concentration in a second or less but we have not verified the role of cathode porosity on oxygen consumption per unit area.

Significance to Biomedical Research and the Program of the Institute:

Because conventional oxygen electrodes consumed substantial current per unit area, they can be sensitive to stirring effects or stagnant layers adjacent to the electrode. In addition to minimizing the stirring effects the new design offers the hope that the oxygen consumption per unit area and therefore sensitivity of a given electrode would be under the control of the manufacturer and not susceptable to user-produced variabilities, that is, sensitivity changes due to membranes being applied more or less tightly over the cathode area. In addition, the rapid response time is a very desirable feature.

Proposed Course:

We are continuing to explore various adhesives to obtain a structure with the desired properties and stable response. When this is achieved we will be able to evaluate the effect of changing cathode porosity on oxygen consumption and response time.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01421-02 LTD

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Critical Care Blood Gas, pH, and Electrolyte Analysis Methods

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

PI: R.E. Steele Physical Scientist LTD NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The structural and operational simplicity of ion-specific electrodes makes them attractive for the determination of certain electrolytes and pH of the serum from critically ill patients. Further understanding of the time response characteristics of these electrodes would benefit this application. We have constructed a unique flow-through pH electrode and determined its time response characteristics using a programmable calculator system for data handling. We found that the sum of 3 exponentials was necessary to fit the time response curves. The time constants were strongly influenced by current flow through the electrode. The longer time constants represented 4 to 8% of the response and had a value of 4 to 12 minutes.

A differential conductometric apparatus for the measurement of small amounts of CO_2 has been constructed. This is an improved version of the continous flow method developed by Steele and Maffly. This work is a first step in the development of a batch method for total serum CO_2 based on this differential conductance principle.

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Objectives:

(1) Construct flow-through ion-specific electrodes for serum electrolytes and pH with small sample volumes.

(2) Study time response characteristics of ion-selective electrodes. The time response of these electrodes has received relatively little study.

(3) Study the effect of serum proteins on the performance of ion-selective electrodes with particular regard to:

(a) ion binding by proteins

(b) activity coefficient changes due to proteins

(c) liquid junction portential changes due to proteins

(d) electrode interferences by proteins.

(4) Extend the application of the differential conductometric method for the measurement of small amounts of CO_2 developed by Steele and Maffly to serum total CO_2 determinations.

Methods Employed and Major Findings:

Steele et al (Proc. San Diego Biomed. Sympos. 14: 469-478, 1975) have used a programmable calculator system to overcome many of the well-known problems of ion-specific electrodes: interferences, drifts, sensitivity (slope) changes, and liquid junction potential changes. This approach will become even more productive as basic knowledge of these electrodes increase. A similar programmable calculator system has been assembled and used for the study of the time response characteristics of a flow-through pH electrode which we have built. The calculator is a Hewlett Packard model 9830A with printer and plotter. An interface bus is used to connect a digital voltmeter and a timing generator. A Keithley model 616 electrometer is used to provide a very high input impedance (greater than $2x10^{14}$ omhs) for glass electrodes. This programmable system provides a versatile means of acquiring, storing, and analyzing data in digital form.

A flow-through pH electrode with a 3 microliter sensing volume has been constructed from a capillary of Corning 0150 glass having 0.040" O.D. and 0.020" I.D. The contact to the outside glass surface (sample is inside) has been made with solid AgCl with a fine Ag wire embedded in it. The electrode has a resistance of $5x10^9$ ohms and a slope of about 58 millivolts per pH unit at 25°C. It was necessary to use the sum of three exponential curves to fit the time response curve of this electrode. When current flow through the electrode was carefully avoided during

solution change (pH8 →pH6), 90% of the response had a 0.05 min. time constant, 6% a 0.9 min. time constant and 4% a 4 min. time constant. After the electrode was shunted with 10^8 ohms, 65% of the recovery had a 0.1 min. time constant, 27% a 1.8 min. time constant and 8% a 12 min. time constant. Comparison with similar data obtained from other electrodes is obviously necessary.

The programmable calculator system has also been used for data handling in the Continuous Flow Method for Determination of Erythrocyte Osmotic Fragility project in this laboratory. Data was taken on-line, stored, processed and plotted. A paper describing this work has been accepted for publication.

A differential conductometric apparatus for the measurement of small amounts of CO_2 has been constructed. This is an improved version of the continuous flow method developed by Steele and Maffly. It can accurately measure CO_2 flux rates of 10 nanomoles per minute with a time constant of about 12 seconds. Batch measurements can be made if the output is integrated. In this way the 13 nanomoles of CO_2 in 0.5 µl of serum can be measured to 1% accuracy. The apparatus is now being used by Dr. Kregenow, NHLBI, LKEM, in the study of ion transport through avian red cell membranes. This apparatus serves as a starting point for the development of higher sensitivity forms of the differential conductometric method and ones specifically intended for measurement of serum total CO_2.

Significance to Biomedical Research and the Program of the Institute:

Ion-specific electrodes are being used in new automated instruments intended for "STAT" determinations of serum electrolytes. Thus, they will frequently be used for samples from critically ill patients. Also, the use of these electrodes in biomedical research is rapidly increasing. As a result, it is important that the advantages and limitations of these electrodes be well understood. A major objective of this project is to advance this understanding and to develop methods of overcoming the limitations particularly in regard to critically ill patients.

Improved CO_2 measurement methods have many clinical and research applications.

Proposed Course:

(1) Make extremely thin pH glass electrodes and study their time response characteristics.

(2) Try sealing thin glass membranes to insulating glass in a vacuum to avoid surface heating and resulting composition changes.

(3) Study time response of reference electrodes.

(4) Try to unscramble the several possible effects of serum proteins on ion-specific electrode performance.

(5) Build a differential conductometric device for serum total CO_2 measurement. Try a porous metal as one conductivity cell electrode to allow CO_2 entry.

Publications:

1. Ito, Y., Carmeci, P., Steele, R.E.: Continuous flow method for determination of erytrhocyte osmotic fragility. Hematology, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE OFFICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01422-01 LTD

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Development of In-Vivo Tissue Culture System - Hybrid Artifical Organ System

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

PI :	D. C. White	Staff Associate	LTD:NHLBI
OTHERS:	J. Pierce	Chief, Lab. of Animal Med. and Surg.	OD:NHLBI
	T. Kolobow	Chief, Section on Pulmonary & Cardiac Assist Devices	LTD:NHLBI

COOPERATING UNITS (if any)

Laboratory of Animal Medicine and Surgery, NHLBI

LAB/BRANCH
Laboratory of Technical Development

SECTION
Pulmonary and Cardiac Assist Devices

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.8	.8	

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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 design and perform in-vivo testing of an implantable tissue culture system. There is evidence to suggest that control of diabetes with insulin does not prevent microangiopathic changes. It is felt that continuous control of glucose levels as normally done by pancreatic B-islet cells can prevent the above mentioned changes. There is interest in developing a system where pancreatic B-islet cells can be grown in a chamber that is perfused with arterial blood. These cells are actually separated from the blood path by a microporous membrane, across which glucose diffuses. Sensing the level of glucose, the cells then release an appropriate amount of insulin that then diffuses across the membrane back into the blood stream. Perhaps a more general use of this system in research would be the ability to grow previously difficult to grow cells,and if desired monitor the effects of their metabolic products on the animal while keeping the cells separated in a growth chamber.

Project Description:

Currently, there is no truly adequate device for growing cells in a chamber that is perfused with blood. Devices similar to artificial kidneys have been used in this manner in the past. A major objective of this report is to design and test a device specifically made for this purpose. After this is accomplished, then the requirements for cell culture will be investigated. Also long-term biocompatibility will be evaluated.

Methods:

a) Device Fabrication

Utilizing the resources of the Laboratory of Technical Development an implanted tissue-cell culture system capable of being perfused with arterial blood has been developed. The current system is made of silicone rubber, polyurethane, and polycarbonate. The device is constructed so that blood flow is directed into all areas of potential stagnation. The growth chamber is separated from the blood path by a 0.8 micron microporous polycarbonate membrane. Samples can be taken from the growth chamber.

b) Studies of Tissue Growth

The device is implanted in sheep and dogs and perfused with blood flowing at 640 cc/min. in a subclavian artery to cephalic vein shunt. Before pancreatic B-islet cells are actually implanted, thyroid and parathyroid tissue will be implanted because it is also endocrine tissue and it is more readily available. Also its function is easy to monitor with serum calcium and thyroxin levels.

Major Findings:

a) A suitable design for the above mentioned purpose has been developed.

b) Surgical methods for cannulation and implantation have been established.

c) Cations and amions have been measured to diffuse to approximately one-half of normal serum values in about 45 minutes. There is complete equilibration by, at least, 5 hours.

d) Blood gas values have been measured to make major shifts toward equilibrium in approximately 20 minutes.

e) Animals tolerate the implantation and shunt flow. There has been no infection.

f) Proper levels of oral anticoagulation are being defined.

Significance of Project to the Institute:

The development of the vasular device as a potential hybrid artificial endocrine pancrease has already been explained.

As a more general research tool this system might allow for the growth of previously difficult to grow cells, while at the same time keeping the cells separate from the animal.

Proposed Course:

1. Continue refining of the design.

2. Find suitable tissues for implantation. Adult and fetal thyroid and parathyroid tissue will be used eventually. Serum thyroxine and calcium will be used to monitor function of cellular activity.

3. Possible collaboration with the Joslin Clinic, Boston for source of B-islet cells.

4. Continual monitoring long-term biocompatibility.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01423-01 LTD

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Biophysical Instrumentation for the Study of Protein Dynamics

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

PI:	R.L. Berger	Chief, Biophysical Inst. Section	LTD NHLBI
	B. Balko	Research Physicist	LTD NHLBI

COOPERATING UNITS (if any)

Biomedical Engineering and Instrumentation Branch, NIH

LAB/BRANCH

Laboratory of Technical Development

SE

Section on Biophysical Instrumentation

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	.5	.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

We have designed and built a Mossbauer Effect apparatus which can be used to perform transmission, scattering, and selective excitation double Mossbauer (SEDM) experiments. We have the flexibility for several modes of operation using ultra stable triangular, rectangular and trapezoidal wave form generators. The rectangular and trapezoidal are position coupled to the drive for stability. These instruments with the highest energy resolution available are being developed to study oxygen binding to the hemoglobin tetramer and the α and β sites individually and to investigate differences between normal Hb, sickle cell(HbS) and Thalassemia blood. We have also developed computer programs to analyze the Mossbauer data using the super-operator formalism. This gives us the most powerful mathematical technique available for studying the interaction of the ion in the active site with its environment.

Project Description:

Objectives:

The objectives of this project are to develop new instrumentation, data handling techniques, mathematical methods, and models of the dynamics of energy exchange, conformational change and other dynamic phenomena involved in the reaction of proteins with their coenzymes and substrates. In particular to develop methods and instruments to study in collaboration with other laboratories, the changes occuring in the proteins hemoglobin and Lactate Dehydrogenase as they react, in the first instance with respiratory gases and small molecules and the second with coenzyme antilogs and substrates particularly with acytlpyridine-NADH and pyruvate.

Model reactions will be studied with especially prepared hematin compounds using replacement and pyrrole ring substituted compounds as well as naturally occuring variants in an attempt to understand the fundamental relations occuring in the hemoglobin reactions. Of particular interest in this study are the factors controlling the conformational change that occurs on oxygenation therefore, instrumentation to observe these changes which occur in the nanosecond to picosecond time domain will be developed. While hemoglobin is a model for a tetrameric molecule whose subunits interact to produce total protein molecular changes resulting in bonding energy changes at the active site, Lactate dehydrogenase is tetrameric enzyme in which there is no subunit interaction but instead a specific piece of the protein, i.e. 13 amino acids of each monomer at the active site shift upon interaction with the coenzyme to produce a new enzyme surface that can react with a specific substrate.

The understanding of this structional change, which again occurs very fast is of importance to both a fundamental understanding of the reactions and pharmocological reaction of vaious inhibitors, etc. Where appropriate, analytical methods are developed for research and clinical applications.

Methods Employed:

The methods employed are those of resonance, relaxation and picosecond spectroscopy. These might include the development of a new type of Mossbauer spectrometer, Laser, Temperature, Pressure, Electric Field, Magnetic field displacement relaxation instruments and the development of a new picosecond Laser spectrometer especially suited to this problem.

Major Findings:

The Mossbauer Effect (Me) is the recoilless emission and subsequent recoilless absorption of nuclear gamma radiation. The importance of

the ME to the investigation of biological processes lies in the fact that it involves radiation of the highest energy resolution available. This allows us to measure very small energy differences. Because the absorption cross section of this radiation in the Mossbauer isotope is six orders of magnitude greater than the next competitive process we can perform measurements on small amounts of material.

The effect can take place only under special conditions which occur in about 100 isotopes. The only isotope of iron that can be utilized in Mossbauer work is Fe^{57} which has a natural abundance of 2.17%. Isotopic enrichment therefore, plays an important role in these experiments.

The development of the selective excitation double Mossbauer (SEDM) technique, to study biological samples by utilizing the high energy resolution available is presently being carried out. Most Mossbauer experiments are performed in the transmission geometry in which one measures the resonant absorption cross section of the material. Such experiments have yielded valuable information about inorganic and organic materials.

We have built and tested four ultra stable special function generators designed to modulate the Mossbauer source emissions in a very specific way.

With these function generators and the two Doppler modulators (Ranger Electronics) we have a system for performing both normal Mossbauer transmission experiments and SEDM experiments. We have tested the linearity of the drives electronically and by taking Mossbauer spectra.

Proposed Course:

The SEDM system will be utilized to study the relaxation phenomenia of Ferrichrome A (specially prepared for us by Neilands, Berekely). This will become the calibration standard and will be studied from 4^{o} K to 320^{o} K.

The important information that can be obtained from the Mossbauer effect for our purposes, is the rather detailed knowledge of the state of the iron ion. Because the environment of the ion changes during biological and chemical processes and the state of the iron does likewise these changes are reflected in the Mossbauer spectrum.

The Mossbauer effect has previously been used to study heme proteins. This relatively early work was largely restricted to the interpretation of the Mossbauer spectra based on the development of appropriate nuclear spin Hamiltonians, and did not relate directly to biological functions. Utilizing enriched model compounds of heme-base complexes prepared for us by Traylor (UCSD) we will investigate energy exchange and relaxation

in oxygen binding to Ferrous hemes. Natural, enriched and modified heme, hemoglobin will then be examined as a model system to study, in collaboration with Clinical hematology, normal and patholgic hemoglobin to determine details of the oxygen reaction with α or β units and specifically the effect of small molecules, effectors, or anti sickling agents on these systems. Laser T-Jump experiments are planned using our present Dye laser and a Picosecond laser spectrometer is under consideration.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01424-01 LTD

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (30 characters or less)

A Centrifuge for the Continuous Harvesting of Blood Cellular Components

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

PI:	T. Kolobow	Chief, Pulmonary & Cardiac Assist Devices	LTD NHLBI
	Y. Ito	Visiting Scientist	LTD NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Technical Development

SECTION

Pulmonary and Cardiac Assist Devices

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
¼	¼	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A flow-through centrifuge without rotating seals was recently described for on-line plasmapheresis of whole blood. We have redesigned the separation chamber to permit continuous plateletpheresis, leukopheresis and plasmapheresis.

The new separation chamber has a single narrow spiral channel (3.5 cm high, 0.45 cm wide, and 100 cm long with a total prime volume of 180 ml) with terminals for the blood inlet and packed RBC outlet, and plasma, WBC and/or platelet outlets. Blood cells sediment in a radially acting centrifugal field (100-340 x g) along a distance of 0.45 cm. In this spiral configuration, blood flows "uphill" against a "g" force gradient which forces heavier cells to travel in opposite direction to plasma and lighter cells (countercurrent flow), each fraction being continuously harvested through the respective terminal.

The device was tested at up to 400 ml/min blood flow rate. Preliminary experiments showed highly efficient separations of plasma, platelets and lymphocytes from the whole blood.

PHS-6040
(Rev. 10-75)

Objectives:

It is the purpose of this project to design and to test a centrifuge system that can fractionate blood cellular components with less cellular damage than using currently available machines.

Frequently, whole blood is withdrawn from donor patients for the sole purpose of obtaining just one blood component, such as red blood cells, granulocytes, lymphocytes, platelets, or plasma.

Methods Employed and Major Findings:

In designing the blood separator we have made use of a recently described method that does not use sliding (rotating) seals to provide for continuous flow of fluids to and from a spinning centrifuge rotor. The blood separation chamber spirals toward the axis of the bowl in a narrow channel. Blood enters peripherally, and travels along the channel towards the center of axis of rotation. Heavier blood cellular elements sediment in the g-field against the wall of the separation chamber, and are progressively displaced outward toward a higher g-field; plasma, and lighter, not yet sedimented cellular components, are displaced inwards towards a lower g-field by flow forces. The net result of the spiral configuration is plasma (and unsedimented cells) flowing in one direction, and sedimented cells flowing in the opposite direction - all in the same narrow channel. Plasma and sedimented cells are then harvested by proper positioning of the pump lines. Because of this and the considerable total length of the spiral, the blood-plasma interface is exceptionally easy to locate and to adjust. In addition, the spiral shape of the centrifuge bowl avoids packing of cells; rather, all sedimented cells are pushed towards respective outlets for harvesting, or for return to the donor (or patient).

We have tested this blood separator in lambs at up to 400 g, and at blood flows to 400 ml/min. We were able to concentrate RBC, and separate plasma, lymphocytes, granulocytes, and platelets. Only heparin has been used in these studies, at relatively low concentrations. We did not observe platelet aggregation. These findings point to superior blood compatibility of the apparatus from both absence of rotating seals, as well as from use of silicone rubber as the only blood contacting surface.

Significance to Biomedical Research and the Program of the Institute:

The blood separator we have designed is a very versatile system for blood cellular element harvesting, fractionation, plasma separation, total body plasma exchange, and for blood banking in general. The whole system is small and is suitable to separate either large volumes, or it can be adapted to the continuous separation of very small volumes (such as ex vivo separation of rabbit blood).

Separation of blood elements has numerous applications to biomedical research and clinical medicine. Preliminary data have demonstrated that the new system reduces morbidity to the donor, as well as enhances quality of the harvested product.

The economics of human blood suggests that we reduce waste, and be specific in our needs. We believe this cell separator represents a step in this direction.

Proposed Course:

We are at present optimizing the blood centrifuge for blood cellular fraction separation.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01425-01 LTD

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Preparative Countercurrent Chromatography with a Slowly Rotating Helical Tube

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

PI: Y. Ito Visiting Scientist LTD NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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)

A simple preparative-scale countercurrent chromatographic scheme was introduced. The scheme uses a helical column slowly rotating in the gravitational field while solvent is eluted through the column by means of rotating seals. Introduction of a two-phase solvent system in the rotating column distributes each phase to occupy nearly half the volume of every turn of the column. Consequently, elution of either phase through the column results in a high efficiency partition process of solutes between the two phases while the eluate is continuously monitored and fractionated. Using a short column, optimum operational conditions were investigated with respect to the column angle, rotational speed, and flow rate for two typical two-phase solvent systems. Under the optimized conditions the capability of the scheme was demonstrated on preparative-scale separations (10 ml sample size) of DNP amino acids and peptides.

Project Description

Objectives: Development of a simple countercurrent chromatographic scheme for preparative-scale separations.

Methods Employed and Major Findings

Principle:

The scheme uses a helical column slowly rotating about its own axis in the gravitational field. When the helical column contains an equilibrated two-phase solvent system, the slow rotation of the helical column distributes each phase to occupy approximately half the space of each turn of the column. Once this dynamic equilibrium is reached, further rotation results in mixing of the phases in each turn of the column without changing the overall phase distribution pattern. Consequently, continuous countercurrent flow is produced by pumping the mobile phase through the column filled with the stationary phase. The mobile phase then replaces the stationary phase with about half the volume in each helical turn and is finally eluted through the column. Thus, solutes introduced in the column are subjected to an efficient partition process between the two phases and are separated according to their relative partition coefficients.

Apparatus:

A helical column is mounted around a hollow rotary shaft (60 cm long) which is equipped with a rotating seal at each end. The rotary shaft is driven by a motor through a pair of pulleys coupled by a toothed belt. The frame holding these elements can be positioned at a desirable angle with respect to the gravity by rotating and tightening it against the standing support. The solvent is pumped through the first rotating seal into the column and the eluate collected through the second rotating seal is continuously monitored with an LKB Uvicord III at 280 nm before fractionation. Each column unit is prepared by winding Teflon tubing (2.6 mm i.d. and 5 m long) onto a plastic pipe (1.25 cm o.d. and 50 cm long) to make about 100 turns. A long column is made by connecting a desirable number of the column units in series.

Phase Retention Studies:

Two-phase systems were selected, $CHCl_3/CH_3COOH/0.1N$ HCl (2:2:1) for DNP amino acid separation and n-BuOH/CH_3COOH/H_2O (4:1:5) for peptide separation. Each phase mixture was equilibrated at room temperature and separated before use.

Using a single column unit, the retention of the stationary phase was measured under various conditions with respect to column angle, rotational

speed and flow rate. In each two-phase system both upper and lower phases were tested as the stationary phase. In order to study the phase distribution and two-phase flow pattern, the stationary phase was lightly colored with a dye which partitions almost entirely to the same phase. The column was first filled with the mobile phase and about 5 ml of the colored stationary phase was introduced into the column which was held vertical so that the stationary phase was completely separated from the mobile phase. The length of the column occupied by the colored stationary phase was measured as A cm. When the column was set to the desired angle, rotated and eluted with the mobile phase at the desired rates, the dynamic equilibrium was reached and the length of the column containing the colored stationary phase was measured as B cm. The retention % of the stationary phase, R, was then calculated as R = A/B x 100.

Satisfactory phase retention of over 40% was observed in a wide range of column angle, revolutional speed and flow rate in both two-phase solvent systems. Overall results indicated that the retention of the organic phase increases with rotational speed allowing greater flow rate while the retention of the aqeous phase decreases with rotational speed limiting applicable flow rate. In most cases an abrupt decrease phase retention was observed close to the horizontal column position where laminary flow of the aqueous phase changes to droplet flow.

Partition Efficiency Studies:

Test samples were selected for each two-phase solvent system, DNP amino acids for the chloroform phase system and peptides for the n-BuOH phase system. In each test two compounds with suitable partition coefficients were dissolved into either phase and 0.2 ml of the sample solution was introduced into a single column unit containing the stationary phase, followed by elution with the mobile phase under a given set of conditions for column angle, rotational speed and flow rate. The eluate was continuously monitored at 280 nm. The efficiency of separation was estimated by measuring the height of the trough between the two peaks and/or expressed in terms of theoretical plates for each peak according to the formula used in gas chromatography.

Studies on effects of the column angle upon the separation clearly indicated that the horizontal column position gives the highest resolution in both phase systems. Effects of rotational speed and flow rate on separation were investigated using the horizontal column. The results indicated that the highest resolution is achieved by a slow flow rate while the time required for yielding one theoretical plate can be minimized by applying a higher flow rate under similar rotational speed.

Preparative-scale Countercurrent Chromatograms:

Under the optimized operational conditions, preparative-scale separations

were performed with a column consisting of 10 column units using a 10 ml sample size in each separation. Efficiencies of 2,000 to several hundred theoretical plates were observed for DNP amino acid separation on the chloroform phase system and 1,000 to a few hundred theoretical plates for peptide separation on the n-BuOH phase system.

Significance to Biomedical Research and the Program of the Institute·

This simple countercurrent chromatographic scheme yields a high efficiency separation of over 1,000 theoretical plates of a 10 ml sample solution. Because of elimination of the use of solid support, the method provides various advantages over the conventional liquid chromatography such as good recovery, high purity and minimum denaturation of samples, high reproducibility and accurate predictability of the peak location etc. We think that the scheme will be extremely useful for separation and purification of various biological materials.

Proposed Course:

Application of the method to separation and purification of various biological materials.

Publications:

1. Ito, Y. and Bowman, R. L: Preparative Countercurrent Chromatography with a slowly rotating helical tube, Anal. Biochem. 78: 506-512, 1977.

2. Ito, Y. and Bowman, R. L.: Preparative Countercurrent Chromatography with a slowly rotating helical tube. J. Chromatogr. 136 189-198 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01426-01 LTD

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Horizontal Flow-Through Coil Planet Centrifuge

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

PI: Y. Ito Visiting Scientist LTD NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	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)

A new scheme for performing countercurrent chromatography has been developed. It uses a helical column revolving around the horizontal axis of the centrifuge and rotating about its own axis at the same angular velocity. This planetary motion, while eliminating the need for the rotating seals, enables the scheme to utilize the gravitational and/or centrifugal force fields to perform a high efficiency countercurrent chromatography with a variety of two-phase solvent systems. Capability of the scheme is demonstrated on preparative-scale separations with two typical two-phase solvent systems.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: Development of the horizontal flow-through coil planet centrifuge for countercurrent chromatography

Methods Employed and Major Findings

Principle:

The separation column is a helical tube supported horizontally and fixed to a gear that rolls around an identical stationary gear mounted on the central axis of the centrifuge. The feed and return tubes of the column are led through the mobile gear and then tightly supported by the stationary gear. Rotation of the mobile gear around the stationary gear produced planetary motion to the column, that is, revolution around the central axis of the apparatus and rotation about its own axis at the same angular velocity. Thus, during one complete revolution of the mobile gear, the helix rotates twice with respect to the gravity and once with respect to the centrifugal force field introduced by revolution. This planetary motion also prevents twisting the flow tubes as they revolve with the mobile gear, allowing the flow in and out through the rotating column without rotating seals.

A simple mathematical analysis has been made on the motion of an arbitrary point on the column to study the resulted acceleration. It was found that the shape of the orbit greatly changes with β the ratio between the radii of rotation and revolution of the column. At $\beta \leq 0.25$, the orbit is a single circular loop. As the β value increases, it changes into a heart shape and then forms a double loop which approaches a double circle with the large β values. The acceleration acting on the point was expressed by two components, the tangential and radial accelerations with respect to the helical column. The tangential acceleration was found to be independent from β values whereas the radial acceleration is largely affected by β giving always negative acceleration at $\beta > 0.25$. This indicates that the large β values cause uneven phase distribution across the diameter of the tube which may increase mass transfer resistance while the smaller β values produce an additional phase mixing to contribute to the partition. In the light of the above analysis, the test system was designed to provide small β values of less than 0.25.

As described in Project Number Z01 HL Z01 01401 LTD, the two-phase solvent system in a rotating helical column tends to distribute itself in such a way that each phase occupies nearly equal amounts in every turn of the column. Once this dynamic equilibrium is reached, further rotation results in mixing of the two phases without changing the overall phase distribution pattern. Consequently, elution of either phase through the column results in an efficient partition process of solutes between the mobile and the stationary phases within each turn of the column.

The unique capability of the present scheme derives from the fact that the column rotates with respect to both the gravitational and centrifugal force fields. When a large-bore preparative column is eluted with an extremely low-interfacial tension phase system, a slow revolutional speed prevents emulsification of the phases which would cause carry over of the stationary phase. For a small-bore analytical column, a high revolutional speed can create a strong centrifugal force field that allows the two phases to counter-flow through a narrow opening of the column without plug flow. Thus, the present scheme is capable of using a variety of two-phase solvent systems for both preparative and analytical-scale separations.

Apparatus:

The rotary frame of the centrifuge consists of a pair of aluminum plates rigidly linked and driven by the motor around the hollow stationary shaft fixed at the central axis of the centrifuge. The frame holds a pair of rotary shaft symmetrically at a distance of 15 cm from the center of the apparatus. Each rotary shaft is equipped with a mobile gear which engages to the identical stationary gear fixed around the stationary shaft. A short coupling pipe is extended from the aluminum plate of the rotary frame and supported through a bearing by a standing support. The helical column can be mounted on the rotary shaft on either side and the counterweight on the other side to obtain a proper balancing of the centrifuge. The revolutional speed of our test system can be adjusted from 0 to 300 rpm.

The separation column was prepared as follows: A Teflon tube (25 mm i.d. and 5 m long) was coiled onto an aluminum pipe (1.25 cm o.d., 48 cm long) to make a column unit. A long column was made by connecting 10 column units in series with fine Teflon tubes (0.4 mm i.d., 15 cm long). The column was then mounted around the rotary shaft by clamping the ends of each column unit with fuse holders symmetrically arranged around the rotary shaft. The flow tubes of the column were first passed through the center-hole of the rotary shaft and then through the side-hole of the coupling pipe to lead into the opening of the stationary shaft. The moving portion of the flow tubes was lubricated with grease and protected with a piece of silicone rubber tube at each supporting point.

A Cheminnert Metering Pump (Chromatronix) was used for elution and an LKB Uvicord III for monitoring the eluate at 280 nm.

Preliminary Experiments:

Using a single column unit, tests were performed to optimize revolutional speed and flow rate for two typical two-phase solvent systems - chloroform/acetic acid/0.1N HCl (2:2:1) and n-BuOH/acetic acid/water (4:1:5). In each separation, the column was filled with the stationary phase and a sample solution containing a pair of selected samples was introduced in the column

followed by elution with the mobile phase at a given flow rate while the apparatus was spun at a given rpm. The partition efficiency was estimated by measuring the height of the trough between two peaks recorded in the chart.

Separation of DNP-L-glutamic acid and DNP-L-alanine on the chloroform phase system was performed under various flow rates and revolutional speeds. The efficiency rose sharply with revolution from 0 to 10 and 25 rpm where gravity plays a major role in partition as the centrifugal force produced by revolution is negligibly small. When the revolutional speed reached a range of 25 to 50 rpm, the gravity failed to retain a satisfactory amount of the stationary phase against the flow and yet the centrifugal force was not strong enough to compensate the gravity. As a result, the efficiency fell with the depletion of the retained stationary phase. Further increase of revolutional speed up to 300 rpm resulted again in a sharp rise of the efficiency where the centrifugal force plays a major role in partition. Similar results were obtained on separation of L-valyl-L-tyrosine and L-tryptophyl-L-tyrosine with the n-BuOH phase system having quite different physical properties form the former chloroform phase system.

Preparative-Scale Separations:

Under optimum conditions in both gravitational and centrifugal force fields, preparative-scale separations were performed with a long column consisting of 10 column units. A set of DNP amino acids were separated on the chloroform phase system using both organic and aqueous phases as the stationary phase. In each separation the samples were dissolved in the stationary phase and 10 ml volume was charged. The efficiencies were calculated in each peak according to the formula used in gas chromatography. All separations showed over 1,000 T.P. in the early peaks which gradually decreased to several hundred T.P. in later peaks. Although good separations were achieved under a slow revolutional speed using the gravitational field, similar results were obtained at 300 rpm with a remarkably shorter elution time of several hours. Similar results were obtained on separations of a set of peptides with n-BuOH phase system. The efficiencies ranged from 1,000 T.P. to a few hundred T.P.

Significance to Biomedical Research and the Program of the Institute:

As demonstrated in the previous chapter, the horizontal flow-through coil planet centrifuge has a capability of utilizing both gravitational and centrifugal force fields for performing countercurrent chromatography. The unique capability enables the scheme to use a variety of two-phase solvent systems for both preparative-and analytical-scale separations of biological materials.

Proposed Course:

1. Refinement of the apparatus to allow higher revolutional speed for analytical separations.

2. Application to separation and purification of various biological samples for scientific research.

Publications:

1. Ito, Y. and Bowman, R. L.: Horizontal Flow-Through Coil Planet Centrifuge without Rotating Seals. Anal. Biochem. In press.

2. Ito, Y., and Bowman, R. L.: Preparative Countercurrent Chromatography with Horizontal Flow-Through Coil Planet Centrifuge. J. Chromatogr. In press.

ANNUAL REPORT OF THE CARDIOLOGY BRANCH
National Heart, Lung, and Blood Institute
July 1, 1976 through September 30, 1977

The basic areas of experimental interest developed in the Cardiology Branch over the past few years have continued. These relate to the pathogenesis, pathophysiology and treatment of coronary artery disease; molecular mechanisms responsible for the contractile function of the heart; development of noninvasive techniques to assess cardiac structure and function; and the application of multi-disciplinary techniques to define the determinants of irreversible heart failure in patients with valvular disease and patients with cardiomyopathy.

Within this framework, several new research directions have been established this year, as previous projects have been completed. Thus, we have finished our studies on the effects of nitroglycerin in acute myocardial infarction. The conclusions derived from these studies have contributed to the widespread utilization of this drug in clinically occurring acute myocardial infarction. A promising new direction we are exploring relates to the role of platelets and platelet inhibitors in modifying ischemic injury.

Greater emphasis is also being placed on studies designed to define the risk factors predictive of sudden death in patients: 1) following an acute myocardial infarction; 2) with stable coronary artery disease; 3) with valvular heart disease, and 4) with ASH.

Finally, development and application of non-invasive techniques to determine myocardial contractile function continues, but experimental emphasis has shifted from echocardiography to radionuclide cineangiography.

CORONARY ARTERY DISEASE

Pharmacologic Treatment of Acute Myocardial Infarction (AMI)

Considerable interest has centered on the platelet as playing a contributory role in the genesis of atherosclerosis. We hypothesized that the platelet also might play a role in AMI, in that acute ischemia may stimulate local aggregation of platelets which in turn would interfere with coronary collateral flow (CCF). Aspirin (ASA), by inhibiting prostaglandin (PG) synthesis, inhibits platelet aggregation. Hence, we tested the effects of ASA on CCF following acute LAD occlusion. We found that ASA given prior to occlusion produced a small but statistically significant increase in epicardial collateral flow.

ASA has complex effects on the PG system. While a beneficial effect during AMI may accrue from anti-platelet effects of ASA, inhibition of PG synthesis in the vascular wall may have deleterious effects. PG in this location may cause vasodilatation and interfere with platelet aggregation. Hence, we are currently attempting to determine the optimal dose of ASA in AMI. We are also trying to determine whether small doses of ASA will affect platelet aggregation without altering PG synthesis in vessel walls.

Diagnosis and Treatment of Stable Coronary Artery Disease (CAD)

Radionuclide Cineangiography

This past year, in collaboration with the Nuclear Medicine Department and DCRT, we developed a real-time radionuclide imaging system with which we can acquire, immediately analyze, and display, left ventricular (LV) scintigrams in movie format that allow assessment of global and regional LV function during exercise. To assess the accuracy of radionuclide cineangiography (RC) we studied 50 consecutive pts with CAD, including 14 who were asymptomatic during exercise and 15 with normal LV function at rest. Each pt demonstrated at least one new region of dysfunction with exercise. In addition, in every pt ejection fraction (EF) either did not change or decreased with exercise. This contrasts to the normal response to exercise in which EF invariably increases. Sensitivity and predictive accuracy of RC was significantly greater than that obtained in the same pts studied by exercise ECG. Thus RC is probably the most sensitive non-invasive indicator of CAD available.

Coronary Artery Bypass Grafting (CABG) and LV Function

CABG results in sypmtomatic improvement in most pts with CAD. However, little information is available relating to the effects of operation on LV function. Since the most sensitive means to assess LV function in CAD is to evaluate the heart during stress, we studied 16 pts with RC pre and post-CABG. In the large majority, EF abnormalities during exercise improved, as did the number of regional wall abnormalities. Hence, these results are the first unequivocal demonstration that CABG reduces exercise-induced ischemic dysfunction in pts with CAD.

Long Term Graft Patency

Another critical question relating to CABG is how long the grafts remain patent. We studied 20 pts (with 30 grafts demonstrated to be patent 6 months post-op) who had CABG between 1971-1973. We found that over 90% of these grafts were still patent at late study. In addition, 20 pts pre-op were functional class (FC) 3 or 4. Early post-op 16 became FC 1. Of these, 11/16 (69%) deteriorated at least one FC by late study. Progress of disease in ungrafted vessels accounted for symptomatic deterioration in 7/11. Hence, although most pts improve symptomatically following operation and grafts remain patent >5 years, symptomatic deterioration is common in the succeeding years. This most often is due to progression of disease in ungrafted vessels.

Exercise LV Dysfunction, and Nitroglycerin (NTG)

NTG prevents ischemic symptoms in pts with CAD when administered before exercise. However, its effects on exercise-induced LV dysfunction are not known. Therefore, pts with CAD were studied by RC at rest and during exercise, before and after NTG. We found that intense exercise invariably induced LV dysfunction, even in pts who did not develop angina. NTG consistently reduced and often normalized such ischemic dysfunction. The occurrence of exercise-induced ischemic dysfunction in the absence of symptoms may predispose to serious arrhythmias. Our finding that NTG eliminates such dysfunction suggests a potential role of prophylactic NTG in pts with CAD.

Exercise and Collateral Blood Flow (CBF)

Although physical training increases exercise capacity in pts with CAD, it is not clear whether this is due merely to enhanced circulatory efficiency (decreased heart rate and BP response to exercise), or can be attributed to training-induced increase in CBF. To determine the effect of physical training on CBF, dogs were prepared with an ameroid constrictor about the LAD and a 70-90% fixed stenosis of the circumflex coronary artery. Two weeks later, regional CBF was measured at rest and during treadmill exercise. One group of dogs then performed exercise five days/wk for 6 wks, while a second group remained in their kennels. After 6 wks, CBF during exercise in untrained dogs was unchanged from initial exercise values. However, endocardial CBF during exercise in trained dogs was 40% greater than prior to training ($p<.05$). Thus the beneficial effects of physical training in CAD may include an improvement in CBF.

Abrupt Withdrawal of Propranolol

Abrupt withdrawal of propranolol in pts with CAD has been associated with worsening of ischemic symptoms relative to pretreatment status, and with AMI. We examined the influence of abrupt withdrawal of propranolol given to normal subjects upon adrenergically mediated heart rate responses and upon platelet survival. Preliminary results suggest that no hypersensitivity of the beta receptor develops. However, shortening of platelet survival time appears to acompany abrupt withdrawal of propranolol. Such a change might reflect alterations that could reduce myocardial blood flow and predispose to potentially serious complications in pts with CAD.

Prospective Studies on the Natural History of Patients with CAD

Patients with Stable CAD: Retrospective studies on the natural history of CAD have determined that mortality rate can be predicted by the number of diseased coronary vessels and the presence and magnitude of ventricular dysfunction. Using such data ""prophylatic", coronary by-pass operation is being recommended. However, these studies are based on data obtained largely from severely symptomatic pts and may not accurately reflect long-term prognosis in the pt with minimal symptoms. Therefore, pts with only mild to moderate functional disability are being studied by cardiac catheterization, exercise testing, 24-hour ECG tape monitoring, and radionuclide cineangiography. Attempts will be made to determine prognostic indices. If high and low risk subgroups can be identified, then more rational decisions can be made regarding which pts should be considered candidates for prophylactic operation. To date, 58 pts have been admitted to study, with an average follow-up of 16 months. There has been only 1 death, and 8 pts have become more symptomatic, necessitating operation.

Patients Recovering from AMI: Pts recovering from AMI are at relatively high risk of sudden death during the first 6 mos after discharge from hospital. More accurate identification of high risk pts might permit selection of pts for aggressive medical and surgical therapy to prolong life. To this end, we have initiated a study in which post-MI pts will be studied immediately prior to hospital discharge by 1) 24-hour ECG monitoring, 2) exercise ECG for arrhythmias, and 3) radionuclide cineangiography at rest and during exercise to determine LV function. Pts will be followed for one year thereafter to determine clinical and functional outcome.

HYPERTROPHIC CARDIOMYOPATHY (ASYMMETRIC SEPTAL HYPERTROPHY - ASH)

In the past few years we have considerably increased our understanding of this disease and its broad clinical and pathophysiological spectrum. It is now apparent that LV outflow obstruction is only an occasional manifestation of the disease. The basic abnormality is a cardiomyopathy characterized by an asymmetrically hypertrophied heart that contains bizarre maloriented myocardial cells, most consistently in the ventricular septum. We also showed that the disease is a genetic abnormality, transmitted as an autosomal dominent trait with a high degree of penitrance.

Specificity of Disproportional Septal Thickening (DST) and of SAM This past year we have studied the specificity of certain clinically important anatomic features found in this disease. For example, although DST is the characteristic marker of genetically-transmitted hypertrophic cardiomyopathy (HCM), we have found by echo and at necropsy that DST can occur in pts with other underlying cardiac lesions. DST was present commonly (20%) in pts with pulmonary stenosis and primary pulmonary hypertension. It also occurred, but less commonly, in about 10% of pts with Eisenmenger syndrome, aortic or mitral valve disease, and in about 5% of pts with CAD and systemic hypertension. Absence of disorganized cardiac muscle cells in the septum of pts with DST and rare occurrence of DST in relatives, suggests that DST in these conditions is not usually a manifestation of genetically transmitted HCM.

Systolic Anterior Motion of the Mitral Valve (SAM) is a characteristic feature of pts with HCM and obstruction to LV outflow, and has been considered virtually pathognomonic of HCM. However, we have now studied 5 pts with non-dilated concentrically thickened hearts and LV outflow obstruction. Each of the pts had SAM, which was apparently responsible for the outflow obstruction. Hence, LV outflow obstruction caused by SAM may occur occasionally in pts with concentric hypertrophy, some of whom do not have HCM. The recognition of this is clinically important, especially when operative intervention is contemplated.

DST and Cellular Disorganization in the Developing Normal Heart Since information concerning ventricular wall thicknesses and cellular disorganization in early human development may be relevant to a better understanding of the morphogenesis of genetically transmitted HCM, morphologic observations were made in normal human embryos, fetuses and live-born infants from eight weeks gestation to 20 months of age. We found that DST was present in 94% of embryos and young fetuses, in 64% of fetuses and infants less than 1 week old, and in only 12% of infants greater than 1 week old. Marked cellular disorganization in the ventricular septum, characteristic of pts with HCM, was not a feature of these hearts.

Sudden Death During this past year two studies with rather alarming results relating to sudden death in HCM have been completed. In the first we found 28 pts whose presenting manifestation of HCM was sudden unexpected death. Six of these individuals were competitive athletes. In the second, 8 families were studied in which the occurrence of premature cardiac death due to HCM appeared to be unusually frequent. A total of 69 first-degree relatives were studied; 41 had evidence of HCM and 73% of these died due to their heart disease. Seventeen of the 30 pts who died were less than 25 years

of age. Death was sudden in 22 pts and of these death was the initial manifestation of cardiac disease in 14. These findings suggest that some families may manifest an unusually virulent form of HCM. Prophylactic propranolol therapy or operation is now being considered in the clinical management of asymptomatic or mildly symptomatic surviving members of these families. Attempts also were made to identify features that might have served as predictors of sudden death. Pts with obstruction seemed to be at considerably higher risk. In addition, both studies suggested that death occurred with greatest frequency in pts with a ventricular septum that was considerably thickened (greater than 20 mm at end systole). We have also studied six pts (age 12 to 60 years) with HCM who had transmural myocardium infarction without significant coronary artery narrowing. Clinically, transmural MI presented in these pts either as sudden death, classical MI, or was clinically silent. Although the cause of AMI in these pts is unknown, this study demonstrates another mechanism that can precipitate sudden death in pts with HCM.

Predictors and Mechanisms of Sudden Death Additional studies are being conducted to identify mechanisms responsible for features predictive of high risk for sudden death. In one study, 24-hour ambulatory ECG monitoring (AEM) of pts with HCM was conducted. No pt had ventricular tachycardia (VT) observed on routine ECG. However, on AEM VT occurred in 23% of pts. Sudden death occurred in two pts within two months of AEM. Both had had VT during AEM. We conclude that: 1) arrhythmias frequently occur in pts with HCM, 2) AEM is useful for identifying pts with previously unsuspected VT and 3) VT detected by AEM may identify pts with HCM at high risk of sudden death.

To determine whether conduction abnormalities may contribute to sudden death in pts with HCM, HIS bundle electrograms are being obtained in a wide spectrum of HCM pts, including those with malignant family histories and those with frequent syncope. Although the data are preliminary, we have now identified several pts who have impaired ventricular condition (increased H-V intervals). This finding, in conjunction with the observation that an occasional pt with HCM may spontaneously develop complete heart block, suggests that transient conduction abnormalities may be a factor contributing to syncope and sudden death in this disease.

Results of Operation LV septal myectomy results in marked symptomatic improvement in almost all pts with HCM. However, the effect of operation on exercise capacity and cardiac function has been controversial. We have studied the symptomatic and hemodynamic responses to exercise in 29 pts pre and post-operatively. Exercise duration and peak oxygen consumption achieved during exercise was consistently increased by operation. In addition, a significant increase in cardiac output during maximal exercise accompanied this performance. Our results suggest that while several mechanisms probably contribute to symptomatic improvement following operation, enhanced cardiac performance plays an important role in most pts.

We are now in the process of analyzing survival statistics in operated pts. The study group comprises 123 consecutive pts who have been followed for more than one year post-operatively. Mean follow-up has been four years, with the original pts operated upon over 15 years ago. Operative mortality

is 7%. Of the pts surviving operation 10% subsequently died cardiac-related deaths; 6% were sudden and 4% died with congestive heart failure. The average annual mortality, exclusive of operative deaths, has been 2.6%. Hence, operative mortality is low and, athough operation does not eliminate the possibility of subsequent death, such events are relatively uncommon. These results, in addition to further analyses of the data which are in progress, will provide the background data necessary to make judgments regarding the wisdom of prophylactic operation in certain subgroups of pts with HCM.

VALVULAR HEART DISEASE

Elucidation of the Determinants of Irreversible Myocardial Failure

We previously completed a retrospective study of long-term survival in pts operated on for aortic regurgitation (AR). Although absolute heart size pre-operatively did not influence long-term postoperative survival, change in heart size, as assessed over the first 4-6 months following operation, did. Thus, 85% of pts operated upon for AR whose cardiac thoracic ratios decreased post-op survived six years, as compared to only 43% of pts whose heart size was unchanged or increased. This prompted a prospective, multi-disciplinary study to define 1) whether a particular grouping of pre-operative functional derangements leads to poor post-operative results, and 2) what type of derangements can be reversed or improved by operation. Studies include exercise testing, echocardiographic and hemodynamic studies, radionuclide evaluation, and 24-hour ECG tapes for arrhythmias. Although results are still preliminary, several observations are of interest. First, pts with low echo ejection fractions (EF), who also had higher LV end diastolic pressures and lower forward cardiac indices, exercised longer than those with normal ejection fractions. Thus, simple exercise testing is not an accurate method for identifying LV dysfunction in pts with AR. Second, echo data suggest that pts with reduced EF pre-op have a high incidence of early (immediate to 6 months post-op) and late (over 6 months) post-op mortality. Thus, 53% of pts with low EF died during follow-up early or late post-operatively, as compared to only 14% with normal EF.

The preceding information is of help in identifying those pts with a poor post-operative survival. However, the clinical challenge is to identify the pt before he/she enters this high risk subgroup. We are therefore evaluating the potential of stress cine-scintigraphy in detecting early evidence of impaired LV function. We have studied pts with hemodynamically severe AR at rest and during supine bicycle exercise. In the majority of pts studied, EF was normal at rest. However, EF decreased during exercise in all AR pts who were symptomatic enough to be considered operative candidates. It also decreased in three of six asymptomatic pts studied. Hence, in pts with AR 1) exercise-induced decrease in LV function precedes the development of symptoms in many (and perhaps most) pts; 2) LV function becomes abnormal with exercise before changes occur at rest. Since abnormal LV function at rest is associated with poor long-term survival after operation, periodic radionuclide assessment of LV function during exercise may be of unique value in determining optimal timing of operation in pts with AR. Similar studies are being conducted in pts with other types of valvular disease.

The Section on Molecular Cardiology has conducted research on cardiac contractile proteins, smooth muscle contractile proteins, and non-muscle contractile proteins.

Cardiac Contractile Proteins

The role of cardiac myosin phosphorylation must differ from that of smooth muscle and non-muscle myosin phosphorylation. Studies in this laboratory indicate that partial phosphorylation (30%) of cardiac myosin does not affect the actin-activated ATPase activity or the K+EDTA and Ca^{2+} activated ATPase activity. It is possible that a major effect will only be observed after more than 50% of the myosin light chains are phosphorylated, or that another protein or proteins such as troponin-tropomyosin are required. Studies are presently underway to answer these and other questions about the role of cardiac myosin phosphorylation.

Smooth Muscle Contractile Proteins

Vas deferens myosin has been isolated in a phosphorylated and non-phosphorylated form. The phosphorylated myosin ATPase activity can be activated by actin but the non-phosphorylated form cannot be activated.

The role of Ca^{2+} in actin-myosin interaction of smooth muscle is indicated from two observations: a) the kinase responsible for phosphorylating smooth muscle cells requires Ca^{2+} for activity, b) purifed phosphorylated smooth muscle myosin (free of kinase and phosphatase) has a higher actin-activated ATPase activity in the presence of Ca^{2+} than EGTA. When smooth muscle cells are placed in culture and evidence loss of differentiation, Ca^{2+} dependent kinase loses its Ca^{2+} requirement. Present experiments are directed toward understanding the biological significance of this loss of Ca^{2+} sensitivity.

Contractile Proteins from Non-Muscle Cells

a) Myoblasts - rat prefusion myoblasts were found to contain a myosin light chain kinase which regulated the actin-activated ATPase activity of proliferative myoblast myosin. Only the phosphorylated species could be actin-activated. The enzyme, similar to other non-muscle kinases, does not require Ca^{2+} for activity. Preliminary experiments indicate that following fusion the myosin kinase activity decreases markedly and perhaps disappears altogether. The possible re-emergence of a new Ca^{2+} dependent enzyme following sarcomere formation is under investigation. Other studies on non-muscle contractile systems include: a) the isolation of a myosin light chain kinase and phosphatase from rabbit macrophages. The possible role of the phosphorylating system in regulating phagocytosis and cell motility will be studied, b) the isolation of a myosin kinase from HeLa cells. The possible role of myosin phosphorylation in regulating mitosis will be investigated, c) the isolation and partial purification of a phosphatase that dephosphorylates platelet myosin derived from platelets. The dephosphorylation of platelet myosin by this phosphatase was shown to decrease the actin-activated ATPase activity of myosin, and hence reverse the effect of phosphorylation.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01645-04 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Contractile Proteins from Proliferative and Post-fusion Myoblast cells.

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

PI:	S. Scordilis	Guest Worker	CB	NHLBI
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Laboratory of General and Comparative Biochemistry, NIMH, NIH.

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Section
Molecular Cardiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	.75	.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 actin-activated myosin ATPase activity of myosin isolated from proliferative myoblasts is dependent on the phosphorylation of the myoblast myosin. The kinase responsible for this phosphorylation is not dependent on Ca^{2+} for its activity. Preliminary experiments suggest that following cell fusion the kinase initially disappears. It may re-emerge following sarcomere formation in a Ca^{2+} dependent form.

PHS-6040

Project Description: Rat proliferative myoblasts grown in culture were used in these experiments. These cells are derived from a Yaffe cloned line, L-5 810. Both myosin and myosin light chain kinase were prepared from these cells. Myosin light chain kinase, similar to the kinase isolated from platelets, was found not to be dependent upon calcium for its activity. This differes from the kinase isolated from both smooth muscle and skeletal muscle cells. Both phosphorylated and non-phosphorylated proliferative myoblast myosin was examined with respect to its actin-activated ATPase activity as well as the ATPase activity of the myosin measured in 0.5 M KCL. These studies have shown that phosphorylation of proliferative myoblast myosin is a pre-requisite for its actin-activation. There was no effect of phosphorylation on myosin ATPase activity measured at high ionic strength. These findings, similar to those previously found for platelet myosin, suggest that phosphorylation of myosin may be an ubiquitous mechanism for controlling actin-myosin interaction in non-muscle cells. Present studies are being carried out to see what effect the development of sarcomeres has on a) the calcium requirement of the kinase and b) control of actin-activation of the myosin ATPase activity by phosphorylation.

Detailed studies carried out with the kinase isolated from proliferative myoblast cells showed that it was cepable of phosphorylating isolated myosin light chains from a number of sources. However, the rate of phosphorylation differed in the following order: platelet light chains > cardiac light chains > skeletal muscle (fast) light chains. The opposite order was observed when the skeletal muscle kinase was used in place of the myoblast kinase.

Studies with astrocytic neuroglial cells grown in culture (both the wild type and the transformed cells) show that these cells contain the contractile proteins actin and myosin and the enzyme, myosin light chain kinase.

Publications:

1. Scordilis, S.P., Anderson, J.L., Pollack, R., and Adelstein, R.S.: Characterization of the myosin phosphorylating system in normal murine astrocytes and derivative SV40 wildtype and A mutant transformant. J. Cell. Biol. (in press).

2. Scordilis, S.P. and Adelstein, R.S.: Myoblast myosin phosphorylation is a pre-requisite for actin-activation. Nature (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01661-02 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
The Natural History of Coronary Artery Disease in Mildly Symptomatic Patients

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

PI:	K. M. Kent	Head, Sec. on Cardiovasc. Diagnosis	CB	NHLBI
Other:	D. R. Rosing	Senior Investigator	CB	NHLBI
	J. S. Borer	Senior Investigator	CB	NHLBI
	S. F. Seides	Senior Investigator	CB	NHLBI
	M. V. Green	Chief, Applied Physics Sec.	NM	CC
	S. L. Bacharach	Physicist	NM	CC
	D. D. Savage	Clinical Associate	CB	NHLBI
	S. E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)
Dept. of Nuclear Medicine, CC, NIH.

LAB/BRANCH
Cardiology Branch

Section
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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)

Patients with coronary artery disease who are only mildly symptomatic are being assessed to determine if a subgroup of patients who are at high risk of sudden death can be identified. More aggressive therapeutic trials would be justified in such a subgroup of individuals.

PHS-6040

Project Description: The management of patients with coronary artery disease is currently being reevaluated since coronary artery revascularization procedures lead to such excellent symptomatic improvement. However, this operation has not been shown to increase longevity or prevent subsequent myocardial infarction. Thus, the role of this operation in the management of patients who have coronary artery disease but who are only mildly symptomatic is unknown. The only data available in the literature on the natural history of patients with coronary artery disease have been accumulated by centers which have active operative intervention programs. Thus, the patients who make up the "control groups" are highly selected individuals who usually have some complicating process which precludes operative intervention. The present investigation was set up as a prospective study of patients with coronary artery disease who are only mildly symptomatic to determine 1) whether low risk and high risk subgroups can be identified and 2) if a high risk subgroup can be identified, whether the annual mortality is sufficiently high enough to warrant consideration of a randomized study in which the results of medically treated and operatively treated groups are compared.

To date 57 patients have been admitted to this study. An additional 13 patients who were referred because of chest pain were found to have minor wall irregularities of the coronary arteries and were not admitted to the formal study. The following investigations are being performed:

1) Cardiac catheterization, including selective left and right coronary arteriograms and left ventricular cineangiograms. Evaluation of the number of coronary vessels involved and extent of involvement is being determined. Left ventricular function is evaluated from the left ventricular cineangiogram. Vagal responses are assessed by examining the reflex decrease in heart rate in response to increased systolic arterial pressure produced by phenylephrine infusion.

2) Isotope angiography (radionuclide cineangiography). Ten mCi of technetium^{99} labelled human serum albumin is injected and a gamma scan of the heart is obtained. With this procedure, left ventricular function can be evaluated in a noninvasive manner not only at rest, but more importantly, during exercise. It is clear that the status of LV function at rest is an important risk group determinant in patients with coronary artery disease. LV function during exercise may be an even more sensitive index of risk in these patients. Thus, it is possible that this totally noninvasive technique will be important in identifying a high risk subgroup.

3) Exercise studies. Some studies suggest that ECG alterations occurring during exercise stress tests (ST segment elevation or marked depression, premature ventricular beats) may predict which patients with coronary artery disease are at a high risk. Thus, patients admitted to this study are exercised in the upright position on a bicycle ergometer at increasing work loads until 85% of predicted maximum heart rate or angina pectoris occurs. ST segments are measured during and after exercise. The frequency and temporal characteristics of premature ventricular beats also are determined.

4) Ambulatory and sleep electrocardiographic monitoring. Twenty-four electrocardiographic monitoring is performed during the second or third day of admission to determine the presence or absence of premature ventricular contractions during daily activities. During the 24 hour period, sleep electrocardiograms are assessed to determine the extent of sleep bradycardia and sleep arrhythmias. It has been demonstrated previously that vagal mechanisms are deficient in patients after myocardial infarction. The degree of sleep bradycardia is used as an index of vagal tone and to determine if this will correlate with subsequent sudden death.

Of the 57 patients admitted to the study, the average followup is fourteen months. At the present time, there has been one death and seven patients who have become more symptomatic necessitating operation.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01665-02 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Phosphorylation and Regulation of Smooth Muscle Myosin

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

PI:	S. Chacko	Guest Worker	CB	NHLBI
	A. Sobieszek	Visiting Associate	CB	NHLBI
Other:	R. S. Adelstein	Head,Sec. on Molecular Cardiology	CB	NHLBI
	W. Anderson,Jr.	Chemist	CB	NHLBI
	J. M. Miles	Med. Biology Technician	CB	NHLBI

COOPERATING UNITS (if any)
Dr. Chacko on leave from the Dept. of Pathobiology, School of Vet. Med., Univ. of Pennsylvania. He will continue part of this work on his return to that Institution in Sept. 1977.

LAB/BRANCH
Cardiology Branch

Section
Molecular Cardiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	.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)

Phosphorylation of guinea pig vas deferens myosin is catalyzed by a Ca^{2+} dependent kinase. Phosphorylation of myosin is necessary for actin-activation of the myosin ATPase activity. The purified phosphorylated vas deferens myosin has a higher actin-activated ATPase activity in the presence of Ca^{2+} than EGTA When smooth muscle cells were placed in culture one major change in the phosphorylating system was the loss of a requirement for Ca^{2+} by the myosin light chain kinase.

PHS-6040

Project Description: Phosphorylation of guinea pig vas deferens smooth muscle myosin by a calcium dependent kinase was found to be necessary for actin activation of the myosin ATPase activity. Moreover the actin-activated ATPase activity of phosphorylated myosin purified free of kinase and phosphatase was found to be inhibited by the removal of calcium. This suggests two mechanisms for the control of smooth muscle contraction by calcium. The first mechanism is based upon the presence of a calcium dependent kinase and a calcium independent phosphatase in the smooth muscle cell. When the calcium level is raised, a kinase is activated and phosphorylation of smooth muscle myosin results. This permits actin activation of the myosin ATPase activity and hence contraction of the smooth muscle occurs. Lowering the calcium concentration results in an inactivation of the myosin light chain kinase. The phosphatase, which remains active at these low concentrations of calcium, dephosphorylates the phosphorylated myosin and relaxation occurs since actin interaction with unphosphorylated myosin cannot occur. A second method of control is suggested by the in vitro experiments which showed that the actin-activated ATPase activity of purified vas deferens myosin is inhibited by lowering the calcium concentration. This suggests an alternate path of smooth muscle relaxation without dephosphorylation of the myosin. Whether such a mechanism is operative for all smooth muscle cells is presently under investigation. Present studies in the laboratory are directed for the comparison of the phosphorylating systems of turkey gizzard, pig stomach and guinea pig vas deferens smooth muscle.

Recently, we have compared the phosphorylating system isolated from the guinea pig aorta to aortic cells that have been placed in culture. Preliminary experiments suggest two major differences in these two systems: The first is that after the aortic cells are placed in culture, the kinase which is dependent upon calcium for activity in the intact aorta, looses its calcium dependency. Moreover, the actin-activated ATPase activity of the phosphorylated myosin isolated in the intact aorta cells appears to be higher in the presence of calcium than in the presence of EGTA. This difference is lost when the cells are placed in culture. Future studies will be directed towards understanding the apparent process of dedifferentiation which results in a change in the effect of calcium on the phosphorylating system, and possibly, in the actin-activated ATPase activity of the myosin.

Publications:

1. Chacko, S., Conti, M.A., and Adelstein, R.S.: Effect of Phosphorylation of Smooth Muscle Myosin on Actin Activation and Ca^{2+} Regulation. Proc. Natl. Acad. Sci. USA 74: 129-133, 1977.

2. Adelstein, R.S., Chacko, S., Barylko, B., Scordilis, S.P., and Conti, M.A.: The Role of Myosin Phosphorylation in the Regulation of Platelet and Smooth Muscle Contractile Proteins. In Contractile Systems in Non-Muscle Tissues (Ed.) S.V. Perry, A. Margreth, and R.S. Adelstein, Elsevier-North Holland, New York. 153-163, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01669-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Relation Between Regional Contractile Function and ST Segment Elevation

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

PI:	H. J. Smith	Visiting Fellow	CB NHLBI
Other:	K. M. Kent	Head, Sec. on Cardivasc. Diagnosis	CB NHLBI
	S. E. Epstein	Chief, Cardiology Branch	CB NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Cardiology Branch

Section
Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	.75	.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)

ST segment elevation and decrease in contractile function are both consequences of myocardial ischemia. The present study demonstrates that endocardial ST segment elevation is the most sensitive marker of myocardial ischemia. However, contractile function is the most sensitive index of ischemia that can be measured clinically.

500

Project Description: The relation between regional contractile function (CF; ultrasonic crystals) and ST segment elevation recorded from both endocardial and epicardial electrodes was examined at thoracotomy in dogs with sudden or graded coronary artery occlusion.

After sudden occlusion of the left anterior descending artery (LAD) in 5 dogs, hypokinetic changes were apparent within 10 seconds. However, ST elevation was first observed in both endocardial and epicardial electrograms at 30 seconds, synchronous with the onset of dyskinesia. By 5 minutes after occlusion, endocardial ST elevation (8.6 $\pm$ 1.3 mv) and epicardial ST elevation (7.6 $\pm$ 1.4 mv) were essentially identical.

In 6 dogs, a carotid-to-coronary shunt was created to perfuse the LAD. A significant reduction in normalized velocity of contraction over the first 1/3 of systole (V1/3n; from 10.1 $\pm$ 1.0 to 3.7 $\pm$ 2.5 mm/sec/cm; $p<0.05$) was first observed after reduction of coronary perfusion pressure (CPP) to 40 mmHg ($p<0.05$). Both the end-diastolic segment length and the mean systolic segment length were significantly increased at CPP = 30 mmHg. Significant endocardial ST elevation (11.7 $\pm$ 3.8 mv; $p<0.05$) was observed after graded occlusion and reduction of CPP to 50 mmHg. Epicardial ST elevation was less than endocardial ST elevation at all levels of CPP and was only significantly increased after reduction of CPP to 30 mmHg (9.0 $\pm$ 1.2 mv; $p<0.05$).

These data suggest that endocardial ST elevation may be a slightly more sensitive index of ischemia than is depression of CF which, in turn, is more sensitive than epicardial ST elevation.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01670-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Natural History of Minimally Symptomatic or Asymptomatic Patients with Aortic Regurgitation

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:	J. S. Borer	Senior Investigator	CB	NHLBI
	K. M. Kent	Head, Sec. on Cardiovasc. Diag.	CB	NHLBI
	S. E. Seides	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 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.2	.15	.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)

In order to develop more sensitive indicators of deterioration in cardiac function in minimally symptomatic or asymptomatic patients with aortic regurgitation, serial invasive and non-invasive testing is being carried out. Studies being performed are echocardiograms, graded exercise tests, 24 hour ambulatory monitoring, radionuclide angiograms, and cardiac catheterization.

Project Description: At present, we employ the development of symptoms in patients with aortic regurgitation as the primary indication for proceeding with valve replacement. However, the results of operation are often less than optimal. In order to develop more sensitive indicators of deterioration in cardiac function in minimally symptomatic or asymptomatic patients with aortic regurgitation, thorough clinical and hemodynamic evaluations are being performed in such patients.

At present, 12 patients have had echocardiograms, graded treadmill exercise tests, 24 hour ambulatory monitoring, and radionuclide angiograms at rest and during supine exercise. Eleven patients have undergone cardiac catheterization at some time in their course and four of these have had pulmonary capillary wedge pressure measured during intense supine exercise. All patients have remained clinically stable, although elevated left ventricular end-diastolic pressures at rest, abnormal responses of left ventricular ejection fraction to exercise, and elevations of mean pulmonary capillary wedge pressure to over 25 mmHg during exercise have been identified. One patient also has had frequent ventricular premature beats including occasional episodes of ventricular tachycardia for over 3 years. It remains to be seen, if any, of our markers will predict imminent clinical deterioration. In order to determine the efficacy of this approach, at least 50 patients will be processed and followed prospectively.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01671-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

The Natural History of Aortic Regurgitation in Symptomatic Patients

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:	J.S. Borer	Senior Investigator	CB	NHLBI
	K. M. Kent	Head, Sec. on Cardiovasc. Diag.	CB	NHLBI
	S. F. Seides	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 20014

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)

In order to identify those patients with aortic regurgitation who are at risk for sudden death and in whom deterioration in cardiac function is imminent, serial invasive and non-invasive testing is being carried out. Studies being performed are echocardiograms, graded exercise tests, 24 hour ambulatory monitoring, radionuclide angiograms and cardiac catheterization.

PHS-6040
(Rev. 10-76)

Project Description: In order to identify those patients with aortic regurgitation (AR) who are at risk for sudden death and in whom deterioration of cardiac function is imminent, a thorough clinical evaluation of all symptomatic patients with moderate to severe AR is being undertaken. At present, 15 patients have undergone right and left heart catheterization, 24 hour ambulatory monitoring, graded treadmill exercise testing, and radionuclide angiograms at rest and during supine exercise. The group will be enlarged to at least fifty patients, and they will be followed prospectively. Thus far 10 patients have been operated upon and 2 have died - 1 suddenly at home shortly after operation, and the other in the hospital after operation. There is insufficient data at present to indicate those persons at risk for sudden death, clinical deterioration, or poor operative result; however, increased numbers of ventricular arrhythmias and poorer exercise capacity have been found in these symptomatic patients, when compared to subjects studied who are minimally symptomatic or asymptomatic.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01672-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Establishment of a Computer 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:	D. R. Rosing	Senior Investigator	CB	NHLBI
Other:	R. H. Dailey	Computer Systems Analyst	DMB	DCRT
	G. D. Stoner	Head, Applied Syst.Prog.Sec.	DMB	DCRT
	K. M. Kent	Head, Cardiovasc. Diagnosis	CB	NHLBI
	G. C. Macks	Management Analyst	OD	CC
	S. E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)
Div. of Computer Research and Technology, NIH (Data Management Branch)
Office of the Director, Clinical Center, NIH.

LAB/BRANCH
Cardiology Branch

Section
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.1	.02	.08

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 base is being established for all cardiology patients. Inpatient and outpatient data will include symptom description, physical findings, and X-ray, ECG, catheterization, echocardiogram, and radionuclide angiogram results. The disposition of the patients will also be indicated.

Project Description: In order to permit easier access to data of cardiology patients for clinical and investigative purposes, and to keep better track of their follow-up status, we are in the process of establishing a computerized data base for all Cardiology Branch patients. Included in these files will be data from both outpatient and inpatient visits as well as the identification of procedures and diagnoses generated at other institutions. The inpatient and outpatient data will include symptom description, physical findings, representative X-ray, ECG, radionuclide, and echocardiographic interpretations list of current medications, and disposition of the patient. In addition catheterization data will be entered into the "MIS" system, immediately transferred to DCRT in the form of "purge tapes", and entered into the data bank with generation of a printout of results for the medical record.

At present all forms for the coding and storage of data are in their final stages as is the development of programs for querying of the system. A form for the acquisition of outpatient information has been approved by Medical Records Committee and should be available soon. This form will replace the present narrative manner of outpatient notes and should insure the recording of uniform and consistent information. Data collection will probably begin in the Fall of 1977. We expect that most patients will be entered into the system by the Fall of 1979. Once the program is working, it will be combined with one already in service under the direction of Dr. Charles McIntosh of the NHLBI Surgical Branch.

Publications: None

END

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01673-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Long-term Cardiac Abnormalities after Operative Correction of Tetralogy of Fallot

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:	J. S. Borer	Senior Investigator	CB	NHLBI
	K. M. Kent	Head, Sec. on Cardiovasc. Diag.	CB	NHLBI
	B. J. Maron	Senior Investigator	CB	NHLBI
	S. F. Seides	Senior Investigator	CB	NHLBI
	A. G. Morrow	Chief, Surgical Branch	SB	NHLBI
	S. E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

Surgical Branch

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	.75	.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)

Twenty patients were evaluated 6-19 years after operative correction of tetralogy of Fallot. Abnormalities of right ventricular diastolic dimensions, cardiac rhythm, and right ventricular pressure were detected. Generally, however, cardiac function was excellent in the majority of patients.

508

Project Description: To assess long-term cardiovascular function after correction of tetralogy of Fallor (TOF), 20 patients, 13-44 ($\bar{25}$) years of age were evaluated 6-19 ($\bar{11}$) years after operation. Several abnormalities were detected: 1) Increased RV diastolic dimensions by echo in 13/17; 2) significant ventricular arrhythmias (multiform, >30/hr, or couplets) were present in 24 hr ambulatory monitoring in 11/18; 3) RV peak systolic pressure was 66 ± 13 mmHg in the 10 patients studied during intense upright exercise (EX). Peak RV outflow gradient was 22 ± 12 mmHg. Nevertheless, overall pumping capacity of the heart during intense upright EX was excellent in most patients as evidenced by a normal maximal oxygen consumption in 20/20 patients and a normal cardiac index in 8/10 patients. In addition, LV ejection fractions determined by radionuclide angiograms were normal at rest in 12/14 patients and during intense supine EX in 10/13. Hemodynamic function was not related to the presence of pulmonary regurgitation nor to the age at or time since operation. These results indicate hemodynamic function is excellent in the majority of patients many years after operative correction of TOF. However, the high frequency of serious ventricular arrhythmias merits continued close observation in such patients.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01674-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Adverse effects of ascorbic acid on cardiac function in myocardial iron overload

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

PI:	W. L. Henry	Senior Investigator	CB NHLBI
OTHER:	A. W. Nienhuis	Chief, Clinical Hematology	CHB NHLBI

COOPERATING UNITS (if any)
Clinical Hematology Branch

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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)

An echocardiographic study of 11 patients with iron overload has demonstrated deterioration in the function of the left ventricle of patients following the addition of ascorbic acid to their therapeutic program. Cessation of ascorbic acid resulted in a return toward normal function. As a result, we believe this drug should be used with caution in patients with myocardial iron overload.

Project Description: Ascorbic acid enhances iron excretion in response to the iron chelator, desferrioxamine, and, therefore, this drug combination is being used in many centers to remove iron from patients who require chronic blood transfusions. We evaluated 11 iron-loaded patients receiving ascorbic acid (250-750 mgm po daily) and desferrioxamine (20 mgm/kg IM daily). Before treatment, echocardiographic ejection fraction was normal. Unexpectedly, there was evidence of cardiac deterioration on serial echoes in 8 patients within 1-8 months of initiation of ascorbic acid; a progressive increase in left ventricular dimension (mean increase 18%) accompanied a fall in LV ejection fraction (mean decrease 35%) ($p<0.01$). In 7 of the 8 patients, decrease in left ventricular function was associated with clinical evidence of congestive heart failure and/or an increase in the cardiothoracic ratio on chest x-ray. In 5 of 6 patients in whom follow-up data were available, ejection fraction returned to within 5% of pretreatment value 3 to 12 months after ascorbic acid was stopped. These data, although circumstantial, implicate ascorbic acid as causing adverse effects on left ventricular function in iron-loaded patients. In addition, we documented the development and subsequent reversal of a severe, dilated (congestive) cardiomyopathy in these patients.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01675-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
24 Hour Ambulatory ECG Monitoring of Patients with Hypertrophic Cardiomyopathy (ASH)

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

PI:	D. D. Savage	Clinical Associate	CB	NHLBI
Other:	S. F. Seides	Senior Investigator	CB	NHLBI
	B. J. Maron	Senior Investigator	CB	NHLBI
	S. E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Cardiology Branch

Section
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.1	.07	.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)

Patients with hypertrophic cardiomyopathy (ASH) were studied by 24 hour ambulatory electrocardiographic monitoring to identify their arrhythmias and to assess the possible relation of these arrhythmias to symptoms, presence or absence of left ventricular outflow obstruction, and sudden death.

Project Description: Sudden death is not a rare complication of hypertrophic cardiomyopathy (HCM). To identify arrhythmias occurring in HCM and their possible relation to sudden death, 50 pts with and 20 without LV outflow obstruction had 24 hour ambulatory electrocardiographic monitoring (AEM) after all medications were discontinued. No patient had ventricular tachycardia (VT) observed on routine ECG. However, on AEM VT occurred in 18% (9/50) of obstructed (ob) patients and 30% (6/20) of nonob patients. VT occurred at rest in 13 of the 15 patients. In the group of patients without VT only 15% (6/41) of ob patients and 8% (1/13) of nonob patients had $\geq$50 ventricular premature beats in any hour. Atrial premature beats ($\geq$50 in any hour) occurred in 8% (4/50) ob patients and in 11% (2/18) of nonob patients in normal sinus rhythm. Paroxysmal atrial fibrillation was observed in 2% (1/50) of ob patients and 6% (1/18) of nonob patients. Sudden death occurred in 2 ob patients within 2 months of AEM. Both had had VT during AEM. No patient without VT on AEM has died during the follow-up period (avg. 8 months). We conclude that: 1) arrhythmias frequently occur in patients with HCM. 2) AEM is useful for identifying patients with previously unsuspected VT. 3) VT may identify patients with HCM at high risk of sudden death.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01676-01 CB

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

ECG Abnormalities in Patients with Hypertrophic Cardiomyopathy (ASH)

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

PI:	D. D. Savage	Clinical Associate	CB	NHLBI
Other:	S. F. Seides	Senior Investigator	"	"
	C. E. Clark	Senior Investigator	"	"
	B. J. Maron	Senior Investigator	"	"
	S. E. Epstein	Chief, Cardiology Branch	"	"

COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

Section

Clinical Physiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.1	.1	0

CHECK APPROPRIATE BOX(ES)

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

☒ (a1) MINORS ☐ (a2) INTERVIEWS

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

The relation of symptoms to electrocardiographic findings in patients with obstructive and nonobstructive hypertrophic cardiomyopathy was studied to determine whether the electrocardiogram might be helpful in distinguishing these subgroups.

Project Description: The relation of the electrocardiogram (ECG) to symptoms in 81 obstructed (ob) and 48 (nonob) patients with hypertrophic cardiomyopathy was studied. Abnormalities are summarized below:

ECG	OBSTRUCTED		NON-OBSTRUCTED	
Finding	Symptom +	Symptom -	Symptom +	Symptom -
Abnl ECG	64/67(96)*	14/14(100)	22/24(92)	17/24(71)**
Abnl T	56/67(84)	10/14(71)	19/23(83)	12/22(55)**
LVH	36/46(78)	7/11(64)	9/18/(50)	5/15(33)**
Abnl ST	45/67(67)	5/14(36)	11/23/(48)	4/22(18)**
LA abnl	41/62(66)	5/13(39)	7/20(35)	4/22(18)**
Abnl Q	23/67(34)	5/14(36)	6/23(26)	7/22(32)
LAD	21/63(33)	5/14(36)	3/21(14)	1/22(5)**
RAE	8/62(13)	3/13(23)	2/20(10)	0/22(0)
1^{o}AV B1	6/64(9)	2/13(15)	4/22(18)	1/24(4)
AF	5/67(8)	0/14(0)	2/24(8)	0/24(0)

*Frequency (%)

The Wolff-Parkinson-White pattern, conduction abnormalities, and RAD occurred in <15% of the patients in each subgroup.

Conclusions: 1) Both symptomatic and asymptomatic patients with HCM have a high prevalence of abnormal ECGs. 2) patients with nonob hypertrophic cardiomyopathy who are asymptomatic have a significantly (**$p<.05$) lower prevalence of ECG abnormalities except abnormal Q waves. 3) AF is the only ECG abnormality that occurred only in symptomatic patients with hypertrophic cardiomyopathy.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01677-11 CE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Echocardiographic Comparison of Black and White Hypertensive Patients

NAMES, LABORATORY AND INSTITUTE AFFILIATION [illegible]
PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D. D. Savage	Clinical Associate	CB	NHLBI
Other:	W. L. Henry	Senior Investigator	CB	NHLBI
	J. R. Mitchell	Chief, Sec.on Metabolism		NHLBI
	A. A. Taylor	Senior Investigator	HE	NHLBI
	J. M. Gardin	Guest Worker	CB	NHLBI
	J. I. M. Drayer	Research Fellow		N.Y.Hosp.-Cornell Med. Center
	J. H. Laragh	Master Professor		N.Y.Hosp.-Cornell Med. Center

COOPERATING UNITS (if any)
Section on Metabolism, NHLBI
Cardiovascular Center, New York Hospital, Cornell Medical Center.

LAB/BRANCH
Cardiology Branch

Section
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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)

Echocardiographic findings of black and white hypertensive patients matched for age, sex, level and duration of hypertension were compared to assess whether there was more cardiac anatomic and functional damage in black hypertensives than white hypertensives.

Project Description: There is much evidence documenting the greater prevalence of hypertension and hypertensive heart disease in blacks than whites. There is no evidence, however, that blacks with the same level and duration of hypertension have more cardiac damage than whites. To assess this possibility we used echocardiography (echo) to study 68 black and 68 white hypertensive patients matched for age, sex, body surface area, and level and duration of hypertension. The results are summarized in this table:

	Blacks	Whites
Age; yrs (range)	(47 (21-67)	46 (19-73)
Body surface area; M^2	1.86±.04	1.89±.03
Known duration of hypertension;yrs	7±.9	7±1
Mean art. pressure; mmHg	111±3	110±2
Left atrium; mm	37±.7	36±.7
Aortic root; mm	31±.5	30±.6
LVID diastole; mm	46±.8	48±.6
Wall thickness*; mm	13±.2	13±.3
Mitral E-F slope; mm/sec	97±3	89±4
LV ejection fraction; %	74±1	75±.8

*avg of ventricular septum + post wall

None of the echo parameters for the two groups were significantly different ($p<.05$). We conclude that hypertensive blacks have no greater prevalence of echo abnormalities than whites with a similar level and duration of hypertension.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HL 01678-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Treadmill Exercise Testing of Patients with Hypertrophic Cardiomyopathy (ASH)

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

PI:	D. D. Savage	Clinical Associate	CB	NHLBI
Other:	S. F. Seides	Senior Investigator	CB	NHLBI
	D. J. Myers	Guest Worker	CB	NHLBI
	F. C. Robinson	Guest Worker	CB	NHLBI
	B. J. Maron	Senior Investigator	CB	NHLBI
	S. E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNIT (if any)

LABORATORY
Cardiology Branch
Section
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.1	.05	.05

CHECK APPROPRIATE BOX(ES)

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

X (a1) MINORS ☐ (a2) INTERVIEWS

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

To assess the usefulness of treadmill exercise testing as a diagnostic tool in hypertrophic cardiomyopathy (ASH), obstructed and nonobstructed patients were studied during exercise.

Project Description: To assess the usefulness of treadmill exercise testing (TET) as a diagnostic tool in hypertrophic cardiomyopathy, 49 obstructed patients and 27 nonobstructed patients were studied. All patients had a ventricular septum to left ventricular free wall ratio $\geq$1.3 on echocardiogram (echo). All medications were stopped 36 to 48 hours prior to TET. ST depression (1 to 4mm) developed in 62% (16/26) of obstructed patients and 40% (4/10) of nonobstructed patients with normal coronary arteries on cineangiography or $\leq$36 years old and minimal risk factors for coronary artery disease. Ten per cent (5/49) of obstructed and 19% (5/27) of nonobstructed patients had $\geq$2 ventricular premature beats per min during or post-TET. Two obstructed patients and one nonobstructed patient had 3 beat ventricular tachycardia during or post-TET. Two of 25 (8%) nonobstructed patients had $\geq$3 atrial premature beats per min during or post-TET. Two obstructed patients died suddenly within 2 months of TET. One had only 2 ventricular premature beats during TET; the other had ventricular tachycardia and frequent ventricular premature beats during TET.

Conclusions: 1) obstructed and nonobstructed patients with hypertrophic cardiomyopathy have a high prevalence of ST depression which may mimic that seen in patients with coronary artery disease. 2) Arrhythmias occur not infrequently during and post-TET in patients with hypertrophic cardiomyopathy. 3) Long term follow-up is needed to determine the prognostic usefulness of TET.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01679-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Aspirin-induced Increase in Collateral Flow after Coronary Occlusion in Dogs

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

PI:	N. L. Capurro	Sr. Staff Fellow	CB	NHLBI
Other:	K. C. Marr	Clinical Associate	CB	NHLBI
	R. E. Goldstein	Senior Investigator	CB	NHLBI
	S. E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Cardiology Branch

Section
Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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)

Aspirin has inhibitory effects on platelet function and prostaglandin synthesis. Since alterations in platelet function and prostaglandin production could influence blood flow to ischemic myocardium, we tested the effects of aspirin on coronary collateral flow following acute coronary occlusion in dogs. Aspirin when administered prior to occlusion in the open-chest, anesthetized dog increased epicardial collateral flow at 4 hours after occlusion. The aspirin dose inhibited in vitro platelet aggregation. Thus aspirin given prior to coronary occlusion produced a small but statistically significant increase in epicardial collateral flow.

PHS-6040

Project Description: Aspirin has inhibitory effects on platelet function and prostaglandin synthesis. Since alterations in platelet function and prostaglandin production could influence blood flow to ischemic myocardium, we tested the effects of aspirin on coronary collateral flow (CCF) following acute LAD occlusion (OCC) in 2 series of dogs. In both series, aspirin dose (30 mg/kg, iv) inhibited in vitro platelet aggregation. In series I, effect of aspirin on CCF was determined (microsphere technique) after 30, 80 and 180 min of OCC in closed-chest, sedated dogs. Aspirin was given to 7 dogs and saline to 6 control dogs 13 min after OCC. In series I, CCF was not altered significantly in aspirin-treated or control dogs. In series II, aspirin was given to open-chest, anesthetized dogs 1 hour before OCC; CCF was determined at 5 and 240 min after OCC. In 8 aspirin-treated dogs CCF increased significantly ($p<.05$) from .12 $\pm$.04 ml/min/g (12 $\pm$ 5% of normal zone flow) at 5 min to .20 $\pm$0.5 ml/min/g (20 $\pm$ 7%) at 240 min. In 7 control dogs CCF did not change significantly (10 $\pm$ 6% at 5 min to 13 $\pm$ 7% at 240 min). The aspirin-induced increase in CCF was confined to epicardium (19 $\pm$ 8% at 5 min to 32 $\pm$ 9% at 240 min; $p<.05$). Thus aspirin given prior to OCC produced a small but statistically significant increase in epicardial collateral flow.

Publications: None

INTRAMURAL SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01680-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Microsphere Loss from Infarcted Myocardium: An Important Technical Limitation.

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

PI:	N. L. Capurro	Sr. Staff Fellow	CB	NHLBI
Other:	R. E. Goldstein	Senior Investigator	CB	NHLBI
	R. Aamodt	Chief, Whole Body Counter Sec.	NM	CC
	S. E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)
Nuclear Medicine Department, CC

Cardiology Branch

Experimental Physiology and Pharmacology

LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
	.05	.05

CHECK APPROPRIATE BOX(ES)

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

(a1) ☐ (a2) INTERVIEWS

ABSTRACT (200 words or less - underline keywords)

Fluorescent radioactive microspheres have been used to map the time-course of development of coronary collateral flow, we tested the stability of microspheres in ischemic tissue. Microspheres were injected prior to coronary artery occlusion in dogs and microsphere estimates of flow to the central ischemic zone were compared with microsphere estimates of flow to the normal zone. We found that preocclusion flow to the central ischemic zone was consistantly underestimated by the microsphere technique in dogs sacrificed at 24 hr , 48 hr, or 8 days after permanent coronary occlusion. Thus, microspheres were lost from ischemic tissue. We conclude that microsphere estimates of collateral flow may be inaccurate when occlusion is maintained for 24 hr or more.

PHS-6340

Project Description: Since radionuclide-labelled tracer microspheres (TM) have been used frequently to map the time-course of development of coronary collateral flow, we tested the stability of TM in ischemic tissue. TM (15μ) were injected prior to left anterior descending coronary artery occlusion (OCC) in 19 closed-chest, sedated dogs. The OCC was released at 13 min in 3 dogs (control) but maintained until sacrifice at 24 hr (n=4), 48 hr (n=4) or 8 days (n=8). Control dogs were sacrificed 48 hr after release of OCC. In control dogs pre-OCC central ischemic zone flow/normal zone flow (IZ/NZ) was not significantly different from unity: .93 ± .04 in the epicardium and .96 ± .03 in the endocardium. However, pre-OCC epicardial IZ/NZ was significantly lower in dogs with sustained OCC that were sacrificed at 48 hr (.69 ± .06; $p<.05$ from control) and 8 days (.70 ± .05 ; $p<.05$), but not at 24 hr endocardial IZ/NZ was significantly reduced as early as 24 hr (.68 ± .03; $p<.01$). The magnitude of the apparent discrepancy in endocardial flow in the IZ was the same at 24 hr, 48 hr and 8 days. Thus, TM radioactivity disappeared from the endocardium of ischemic tissues as early as 24 hr after OCC, and from the epicardium by 48 hr after OCC. We conclude that TM estimates of collateral flow may be inaccurate when OCC is maintained for 24 hr or more.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HL 01681-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Disproportionate Ventricular Septal Thickening in the Developing Normal Human Heart

NAMES, LABORATORY AND INSTITUTE AFFILIATION, AND TITLES OF PROFESSIONAL PERSONNEL ENGAGED

PI:	B. J. Maron	Senior Investigator	CB, NHLBI
Other:	J. Verter	Statistician	BB, NHLBI
	S. Kapur	Staff Pathologist	Children's Hospital of D.C.

COOPERATING UNITS (if any)
Biometrics Research Branch, NHLBI
Children's Hospital of D.C. Department of Pathology

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland

TOTAL MANYEARS:
.2

PROFESSIONAL:

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underlined portion)

Disproportionate ventricular septal thickening is characteristic feature of the normal human embryonic heart. The frequency of disproportionate septal thickening becomes less common after birth and subsequent development and particularly uncommon after 2 weeks of age. This occurs because disproportionate period of growth studied by [illegible] at a greater rate than the ventricular [illegible]

PHS-2040

Project Description: A disproportionately thickened ventricular septum containing numerous disorganized cardiac muscle cells is the most characteristic anatomic feature of genetically transmitted hypertrophic cardiomyopahty (i.e., ASH). Since information concerning ventricular wall thicknesses and cellular disorganization may be pertinent to understanding the development of hypertrophic cardiomyopathy, morphologic observations were made in 151 normal human embryos, fetuses and live-born term infants from eight weeks gestation to 20 months of age. Disproportionate ventricular septal thickening (septal-free wall thickness ratio $\geq$1.3) was present in 94% of embryos and young fetuses; in over one-third of these hearts the disproportionate thickening was particularly pronounced (i.e., septal-free ratio $\geq$2.0). Disproportionate septal thickening was also present in 64% of older fetuses and term infants less than one week of age but in only 12% of infants over two weeks of age (each with septal-free wall ratio of just 1.3). Septal-free wall ratio gradually decreased in a curvilinear fashion with increasing gestational age and approximated unity in the newborn infant over two weeks of age. This phenomenon is due to the fact that while both ventricular septal and left ventricular free-wall thicknesses increased directly with age (r=0.92 and 0.96, respectively), septal thickness increased at a slower rate relative to left ventricular free-wall thickness after birth. Marked cellular disorganization in the ventricular septum was not a feature of the hearts studied. Hence, disproportionate septal thickening, typical of patients with hypertrophic cardiomyopathy, is also a characteristic feature of the normal human heart during its earliest stages of development, but is uncommon after the second week of life.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01682-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
"Malignant" Hypertrophic Cardiomyopathy

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

PI:	B. J. Maron	Senior Investigator	CB	NHLBI
Other:	L. C. Lipson	Clinical Associate	CB	NHLBI
	W. C. Roberts	Chief, Pathology Branch	PB	NHLBI
	D. D. Savage	Clinical Associate	CB	NHLBI
	S. E. Epstein	Chief, 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 20014

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)

Premature death occurs with particular frequency in certain families with hypertrophic cardiomyopathy. Such families constitute a high risk subgroup of patients with hypertrophic cardiomyopathy in whom prophylactic medical and surgical treatment may be indicated.

526

PHS-6040
(Rev. 10-76)

Project Description: Eight families in which the occurrence of premature cardiac death due to hypertrophic cardiomyopathy (i.e., ASH) was unusually frequent were identified. A total of 69 first degree relatives were studied; 41 had evidence of hypertrophic cardiomyopathy and 30 (73%) died due to their heart disease. Seventeen of these 30 pts were less than 25 years of age at death. Death was sudden in 22 of the 30 pts and in 14 of these 22 pts sudden death was the initial symptomatic manifestation of cardiac disease. The remaining 8 pts died after chronic cardiac illnesses characterized by congestive heart failure, atrial fibrillation or thromboembolic events.

Hence, premature death occurs with particular frequency in certain families with hypertrophic cardiomyopathy. Such deaths are usually sudden, often occur in previously asymptomatic subjects, and are particularly common in children and young adults. These findings suggest that some families may manifest an unusually virulent expression of hypertrophic cardiomyopathy. "Prophylactic" propranolol therapy or septal myotomy-myectomy should be considered in the clinical management of asymptomatic or mildly symptomatic surviving members of these families.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01683-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Disproportionate Ventricular Septal Thickening in Coronary Artery Disease

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

PI:	B. J. Maron	Senior Investigator	CB NHLBI
Other:	D. D. Savage	Clinical Associate	CB NHLBI
	C. E. Clark	Senior Investigator	CB NHLBI
	W. L. Henry	Senior Investigator	CB NHLBI
	Z. Vlodaver	Research Fellow	Miller Hosp.-St.Paul, Minn.
	J. E. Edwards	Chief, Dept. of Pathology	Miller Hosp.-St. Paul,Minn.
	S. E. Epstein	Chief, Cardiology Branch	CB NHLBI

COOPERATING UNITS (if any)

Miller Hospital, St. Paul, Minnesota.

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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)

About 10% of patients with coronary artery disease in this study have disproportionate septal thickening. This finding appears to be due to three possible etiologies: (1) associated genetically transmitted hypertrophic cardiomyopathy; (2) coronary artery disease itself; (3) scarring and thinning of the posterior left ventricular wall.

528

PHS-6040
(Rev. 10-76)

Project Description: A disproportionately thickened ventricular septum (septal-free wall ratio ≥1.3) can occur in patients with various acquired or congenital heart diseases. However, the prevalence of disproportionate septal thickening in patients with coronary artery disease is unknown. Hence, this study was undertaken to determine: 1) prevalence of disproportionate septal thickening in patients with coronary artery disease; 2) whether disproportionate septal thickening can be secondary to coronary artery disease or is always a manifestation of genetically transmitted hypertrophic cardiomyopathy. Echo or necropsy studies were performed in 152 patients with coronary artery disease. Disproportionate septal thickening was present in 17 (11%) of the 152 patients, including 9% of patients studied at necropsy. Genetic transmission of septal thickening was demonstrated by echo in first degree relatives of 3 of 9 patients with coronary artery disease and disproportionate septal thickening. However, no patient with disproportionate septal thickening studied at necropsy had numerous disorganized cardiac muscle cells in the septum (typical of hypertrophic cardiomyopathy). Thus, genetically transmitted hypertrophic cardiomyopathy may occur in patients with coronary artery disease. However, absence of disorganized cells in the septum of patients studied at necropsy and negative family echo studies in some patients with coronary artery disease and disproportionate septal thickening suggest that nongenetic disproportionate septal thickening may be a consequence of coronary artery disease.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01684-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Myocardial Infarction in Hypertrophic Cardiomyopathy

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

PI:	B. J. Maron	Senior Investigator	CB NHLBI
Other:	W. C. Roberts	Chief, Pathology Branch	PB NHLBI
	D. E. Chenowith	Resident in Pathology	Univ. of Calif. at Irvine
	S. E. Epstein	Chief, Cardiology Branch	CB NHLBI

COOPERATING UNITS (if any)
Department of Pathology - University of California at Irvine
Pathology Branch, NHLBI

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.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)

Transmural myocardial infarction was identified in six patients with hypertrophic cardiomyopathy and normal coronary arteries. Transmural myocardial infarct presented as sudden death, classical infarct or was silent. While left ventricular dilatation is rare in hypertrophic cardiomyopathy, one of the factors predisposing to this unusual feature appears to be transmural myocardial infarction.

530

Project Description: Among 65 patients with hypertrophic cardiomyopathy studied at necropsy, 6 patients (aged 12 to 60 years) had transmural myocardial infarction without significant (>50% reduction in cross-sectional area) coronary arterial narrowing by atherosclerosis. In 3 of the 6 patients clinical manifestations of myocardial infarction were absent, although 2 other patients had cardiac arrests that probably were due to myocardial infarction; in 1 patient myocardial infarction presented in the typical clinical fashion. Two patients had obstruction to left ventricular outflow and 4 did not. In addition to transmural myocardial infarction (involving primarily the ventricular septum), other cardiac findings included: 1) extensive nontransmural scarring (including subepicardial areas) in the left ventricular free wall and ventricular septum (6 patients) and right ventricle (4 patients); 2) Massive (1250 gm) cardiomegaly (1 patient); 3) dilatation of both ventricular cavities (5 patients); 4) narrowed intramural coronary arteries (2 patients). In summary, transmural myocardial infarction may occur in patients with hypertrophic cardiomyopathy in the absence of significant coronary artery disease. Clinically, however, transmural myocardial infarction may present as sudden death, classical myocardial infarction, or may be silent. Finally, while left ventricular dilatation is rare in hypertrophic cardiomyopathy, one of the factors predisposing to this unusual feature appears to be transmural myocardial infarction.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01685-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Association of Secundum Atrial Septal Defect and Atrioventricular Nodal Dysfunction.

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

PI:	B. J. Maron	Senior Investigator
Other:	J. S. Borer	Senior Investigator
	S. H. Lau	Staff Cardiol., Cardiopulmonary Lab.-USPHSH-Staten Island
	A. N. Damato	Chief,Cardiopulmonary Lab.- USPHSH-Staten Island
	L. P. Scott	Chief, Pediatric Cardiol.- Children's Hosp.,D.C.
	S. E. Epstein	Chief, Cardiology Branch

COOPERATING UNITS (if any)
U.S.P.H.S. Hospital - Staten Island

Children's Hospital, District of Columbia

LAB/BRANCH
Cardiology Branch

Section
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.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)

This report describes a family (in three consecutive generations) with the association of secundum atrial septal defect, conduction abnormalities on ECG and syncope. Electrophysiologic studies in selected members of this family showed evidence of atrioventricular nodal dysfunction. Hence, both atrial septal defect and A-V nodal disease may be transmitted as autosomal dominant traits and may occur together or separately in members of the same family.

532

PHS-6040
(Rev. 10-76)

Project Description: Secundum atrial septal defect may occasionally be transmitted as an autosomal dominant trait. Patients with this entity often demonstrate evidence of conduction system abnormalities by electrocardiogram. To define the nature of the conduction system disease in such patients, we studied a family in which five closely related members in three generations demonstrated the combination of atrial septal defect, syncope, and first and/or second degree atrioventricular (A-V) block by electrocardiogram. Three of these five patients had received permanent pacemakers. Three other relatives without syncope also had conduction abnormalities, including one patient with atrial septal defect. Three of the eight family members were studied with His bundle electrocardiography. Two of these three patients had syncope preceding operative closure of an atrial septal defect and one had no history of syncope or evidence of an atrial septal defect: all three had first degree A-V block. At intracardiac electrophysiologic study, each patient had markedly prolonged A-H time (which decreased with atropine), normal H-V time and prolonged A-V nodal refractory periods. Therefore, both secundum atrial septal defect and intrinsic A-V nodal disease may be transmitted as autosomal dominant traits, and may occur together or separately in members of the same family.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01686-01 CB

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Prevalence and Characteristics of Disproportionate Septal Thickening in Systemic Hypertension

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

PI:	B. J. Maron	Senior Investigator	CB NHLBI
Other:	J. E. Edwards	Chief, Dept. of Pathology	Miller Hosp.- St.Paul,Minn.
	S. E. Epstein	Chief, Cardiology Branch	CB NHLBI

COOPERATING UNITS (if any)

Department of Pathology, Miller Hospital, St. Paul, Minn.

LAB/BRANCH

Cardiology Branch

Section

Clinical Physiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.2	.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)

In a study group of 38 patients who died of systemic hypertension without coronary artery disease, disproportionate septal thickening proved to be an uncommon finding (i.e., prevalence of 6%). These instances of disproportionate septal thickening appear to be a secondary manifestation of the left ventricular pressure overload present in hypertension.

534

PHS-6040
(Rev. 10-76)

Project Description: To determine the prevalence and characteristics of disproportionate ventricular septal thickening (septal-free wall ratio of ≥1.3) in a population of patients with severe chronic hypertension, unassociated with coronary artery disease, 33 patients were studied at necropsy. The overall prevalence of disproportionate septal thickening was relatively low, i.e., 2 (6%) of the 33 patients. Septal-free wall ratio in both of these patients was 1.3. Disproportionate septal thickening appeared to be secondary to the left ventricular pressure overload, rather than to coexistent genetically transmitted hypertrophic cardiomyopathy. This conclusion was based on the fact that: 1) numerous disorganized cardiac muscle cells, characteristic of hypertrophic cardiomyopathy, were not present in the ventricular septum of either patient with disproportionate septal thickening and 2) echocardiographic studies performed in first degree relatives of one of the two patients did not disclose disproportionate septal thickening. Hence, disproportionate septal thickening may occur as a secondary manifestation of the left ventricular pressure overload present in patients with systemic hypertension, but this association appears to be relatively uncommon.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01687-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Sudden Death in Asymptomatic Patients with Hypertrophic Cardiomyopathy

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

PI:	B. J. Maron	Senior Investigator	CB NHLBI
Other:	W. C. Roberts	Chief, Pathology Branch	PB NHLBI
	J. E. Edwards	Head,Dept.of Pathology	Miller Hosp.,St.Paul,Minn.
	H. A. McAllister	Chief,Cardiovasc.Pathology	Armed Forces Inst.of Path.
	D. D. Foley	Pathology Resident	Miller Hosp.,St.Paul,Minn.
	S. E. Epstein	Chief, Cardiology Branch	CB NHLBI

COOPERATING UNITS (if any)
Pathology Branch, NIH
Miller Hospital, St. Paul, Minn.
Armed Forces Institute of Pathology, Washington, D.C.

LAB/BRANCH
Cardiology Branch

Section
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.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)

Twenty-five patients with hypertrophic cardiomyopathy, in whom the first symptomatic menifestation of cardiac disease was sudden death, were identified. Each of the patients demonstrated a distinctly abnormal electrocardiogram and moderate to severe ventricular septal thickening, suggesting that these findings may be predictive of sudden death in asymptomatic patients with hypertrophic cardiomyopathy.

PHS-6040
(Rev. 10-76)

Project Description: Sudden death is a recognized complication in symptomatic patients with hypertrophic cardiomyopathy. The occurrence of sudden death in patients with no or transient previous cardiac symptoms presents, however, a particularly challenging diagnostic and therapeutic dilemma. Therefore, 25 such patients with hypertrophic cardiomyopathy in whom death was the first definitive symptomatic manifestation of cardiac disease, were evaluated. Ages ranged from eitht to 49 years (mean 18) and 22 were less than 25 years of age, 19 were male and six were female. Of the 25 patients, 12 died during or immediately after moderate or severe physical exertion. Twelve patients had previous cardiac catheterization; six had no or a small left ventricular outflow tract gradient under basal conditions and six had outflow gradients of $\geq$50 mmHg. Left ventricular end-diastolic pressure was elevated in nine patients. The ventricular septum was moderately to severely thickened ($\geq$17 mm) in all patients. The electrocardiogram was abnormal in each of the 18 patients studied before death. Thus, sudden death may be the first definitive symptomatic manifestation of cardiac disease in some patients with hypertrophic cardiomyopathy. Although we cannot exclude the effects of patient selection in this study group, sudden death occurred not uncommonly in children and young adults and was often related to physical exertion; each patient showed a distinctly abnormal electrocardiogram and moderate to severe ventricular septal thickening.

Publications: None

END

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01688-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Left Ventricular Outflow Tract Obstruction in Patient with Concentric Hypertrophy

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

PI:	B. J. Maron	Senior Investigator	CB	NHLBI
Other:	J. S. Gottdiener	Expert	CB	NHLBI
	W. C. Roberts	Chief, Pathology Branch	PB	NHLBI
	W. L. Henry	Senior Investigator	CB	NHLBI
	S. E. Epstein	Chief, Cardiology	CB	NHLBI

COOPERATING UNITS (if any)

Pathology Branch

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.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)

Five patients with concentric left ventricular hypertrophy, non-dilated hearts and left ventricular outflow tract obstruction (with systolic anterior motion of the anterior mitral leaflet, i.e., SAM on echocardiogram) were identified. Two of these patients had documented evidence of typical hypertrophic cardiomyopathy in their relatives. Hence, left ventricular outflow obstruction may occur in the presence of symmetric or asymmetric septal hypertrophy.

538

PHS-6040
(Rev. 10-76)

Project Description: Patients with typical genetically transmitted hypertrophic cardiomyopathy (i.e., asymmetric septal hypertrophy) may show obstruction to left ventricular outflow under basal conditions or with provocative maneuvers. However, the presence of dynamic left ventricular outflow tract obstruction in patients with concentric ventricular wall thickening (but without abnormalities of the aortic valve) has been less well appreciated.

Clinical and morphologic features of five patients with non-dilated left ventricles and left ventricular outflow obstruction are presented. In each patient peak systolic pressure gradients between left ventricle and systemic artery were measured at cardiac catheterization and ranged from 60-140 mmHg under basal conditions or with provocation. Each patient had echocardiographically documented systolic anterior motion of the anterior mitral leaflet, which was apparently responsible for the outflow obstruction, and concentric left ventricular wall thickening (septal-free wall thickness ratio of <1.3). Two of the five patients had evidence of genetically transmitted hypertrophic cardiomyopathy, as evidenced by disorganized cardiac muscle cells in the ventricular septum or asymmetric septal hypertrophy in first degree relatives. Hence, left ventricular outflow tract obstruction associated with systolic anterior motion of the anterior mitral leaflet may occur in some patients with concentric left ventricular hypertrophy who do not have typical hypertrophic cardiomyopathy.

Publications: None

END

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01689-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Phosphorylation of Cardiac and Scallop Myosins

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

PI: M. A. Conti	Graduate Student	CB	NHLBI
R. S. Adelstein	Head, Sec. on Molecular Cardiology	CB	NHLBI
Other: W. Anderson, Jr.	Chemist	CB	NHLBI
R. Heinen	Summer Student	CB	NHLBI

COOPERATING UNITS (if any)

Dr. Andrew Szent-Gyorgyi (Brandeis University, Waltham, Mass). (Scallop myosin)

LAB/BRANCH
Cardiology Branch

Section
Molecular Cardiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	.75	.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)

Canine cardiac myosin was phosphorylated using the myosin light chain kinase from rabbit skeletal muscle myosin. Incorporation of approximately 0.6 moles of phosphate/mole of myosin into the 20,000 dalton light chain of myosin had very little, if any, effect on the actin-activated myosin ATPase activity.

Using the platelet myosin light chain kinase approximately 0.2 moles of phosphate was introduced into the scallop myosin regulatory light chain.

540

PHS-6040
(Rev. 10-76)

Project Description: Cardiac myosin was prepared from fresh canine ventricles by a high salt extraction (.5M KCl, 15 mM Tris.HCl, pH7.5, 1mM EDTA, 2.5mM DTT) followed by a low salt precipitation (.08M KCl, pH6.3). There was no evidence for endogenous phosphorylation of the cardiac mysoin light chain (20,000 MW). Myosin light chain kinase could not be detected when the myosin preparation was incubated with an isolated fraction of cardiac myosin light chain nor could phosphatase activity be detected upon incubation with ^{32}P-labelled cardiac myosin light chains. Myosin light chain kinase was isolated from rabbit skeletal muscle by an extract (4mM EDTA, pH7.5) of a lyophilized powder of rabbit skeletal muscle actomyosin. The skeletal muscle myosin kinase incorporated 0.6 moles PO_4 per mole cardiac myosin (0.6 moles out of a possible 2 moles of light chain). Cardiac myosin was then purified and freed of the exogenous kinase by gel filtration on Sepharose 4B. The actin-activated ATPase of the purified cardiac myosin (.11 μmoles P_i/Mm myosin/min, 37°C) was unaffected by this extent of phosphorylation, unlike the actin-activated ATPase of platelet myosin which is markedly increased upon phosphorylation.

Further experiments will be carried out to increase the level of cardiac myosin phosphorylation. Cardiac muscle troponin will be added to investigate its effect on myosin phosphorylation.

An isolated fraction of scallop myosin regulatory light chain has been phosphorylated (0.2 moles phosphate / mole light chain) by the myosin light chain kinase isolated from human blood platelets. Endogenous kinase is being investigated in preparations of actomyosin from whole scallops and scallop myofibrils. Future experiments will be directed toward phosphorylating scallop myosin and studying Ca^{2+}regulation of the actin-activated ATPase of phosphorylated vs unphosphorylated scallop myosin.

Publications:

Maron, B. J., Ferrans, V.J. and Adelstein, R.S.: Isolation and characterization of myosin from subjects with asymmetric septal hypertrophy. Circulation Research 40: 468, 1977.

Adelstein, R.S., Maron, B.J., Daniel, J.L., Conti, M.A., Anderson, W. Jr., and Cohen, E.R. The Phosphorylation of Human Platelet Myosin and Human and Canine Cardiac Muscle Proteins. In Second Joint Symposium on Myocardial Metabolism, Sochi, USSR. 73-84, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01690-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Effects of Coronary Artery Bypass Operation on Left Ventricular Function During Exercise

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

PI:	K. M. Kent	Head, Sec. Cardiovasc. Diagnosis	CB	NHLBI
Other:	J. S. Borer	Senior Investigator	CB	NHLBI
	M. V. Green	Chief, Applied Physics Sec.	NM	CC
	S. E. Bacharach	Physicist	NM	CC
	C. L. McIntosh	Senior Surgeon	SB	NHLBI
	D. M. Conkle	Senior Surgeon	SB	NHLBI
	S. E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

Nuclear Medicine Dept., CC
Surgery Branch, NHLBI

LAB/BRANCH
Cardiology Branch

Section
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.1	.75	.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)

Radionuclide cineangiograms were performed at rest and during exercise in patients before and after coronary artery revascularization procedures. Exercise induced myocardial contractile abnormalities were less after coronary revascularization.

542

PHS-6040
(Rev. 10-76)

Project Description: Previously, the effects of coronary artery bypass operation (CABG) on left ventricular function (LVF) have been evaluated only with patients at rest. These studies have demonstrated no consistent improvement post-operatively. However, many abnormalities in LVF at rest are due to irreversible myocardial damage. Furthermore, abnormalities induced only during exercise must occur in potentially viable myocardium, and therefore may be reversed by CABG. Hence, we studied the effect of CABG on LVF during exercise. ECG gated Tcm99 radionuclide cineangiograms were performed preop and 2-4 months postop in 16 consecutive patients with coronary artery disease. Because scintigraphic images of the LV were obtained in movie display format, regional as well as global LV function could be assessed. The 16 patients received a total of 31 grafts. Coronary angiography was obtained in all but one of the patients postoperatively and 80% of the grafts were patent at 6 months. At rest, ejection fraction was $50\pm3\%$ preop and was unchanged postop $50\pm4\%$ NS. However, preop, in 15/16 patients, ejection fraction decreased during exercise with no change in 1 patient (avg decrease 26%, $p<.01$). In contrast, postop ejection fraction increased during exercise in 8/16 patients ($p<.05$) and in each of the remaining 8 patients the ejection fraction was unchanged with exercise or decreased less than preop. Ejection fraction during exercise postop was significantly greater than preop ($51\pm5\%$ vs $37\pm3\%$, $p<.01$). Analysis of regional function during exercise showed that of 31 LV segments that received bypass grafts, 18 demonstrated improved function during exercise postop (12 had become normal); 2 segments developed greater segmental wall abnormalities postop. We conclude that while CABG may have little or no effect on LVF at rest, exercise-induced wall motion abnormalities are improved. Thus, these preliminary results provide direct evidence that CABG causes functionally important increases in myocardial blood flow.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01691-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Cardiac Size and Function in Anorexia Nervosa

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

PI:	J. S. Gottdiener	Expert	CB	NHLBI
Other:	H. A. Gross	Staff Psychiatrist		NIMH
	W. L. Henry	Senior Investigator	CB	NHLBI
	J. S. Borer	Senior Investigator	CB	NHLBI
	M. E. Ebert	Chief,Exp.Therap.Sec.		NIMH

COOPERATING UNITS (if any)
National Institute of Mental Health

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.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)

Nine patients with anorexia nervosa were studied by echocardiography, ECG, maximal treadmill exercise testing and 24 hr taped ECG; 3 underwent Tc^{99m} radionuclide angiography at rest and exercise. Although cardiac performance was unimpaired, 3/4 patients in whom left ventricular mass was below 95% predictive limits had ventricular ectopy. No patient with heart size appropriate for body surface area evidenced ectopy. Repeat echocardiograms in 6/9 patients performed after mean 27% weight gain showed 28% increase in LV mass and 21% increase in left atrial dimension. Cardiac size may increase following weight gain in Anorexia Nervosa. Ventricular ectopy may be present despite normal LV mechanical function.

544

Project Description: Mortality in Anorexia Nervosa is the highest of any psychiatric disease and may be of cardiac origin. To examine cardiac size and function in this condition, 9 patients were studied by echocardiography (echo) and maximal treadmill exercise testing with O_2 consumption (VO_2); 3 patients underwent Tc^{99m} radionuclide cineangiography (RC) at rest and exercise. The presence of ventricular extrasystoles (PVCs) was determined during exercise and from 24 hr ECG tapes. Cardiac performance was unimpaired, as determined by normal echo ejection fraction (EF) (75 $\pm$ 5%), VO_2 (28.3 $\pm$ 8.8 ml/min/kg and normal exercise augmentation of EF by RC. In 4/9 patients, echo left ventricular (LV) diastolic dimension (Dd), wall thickness or mass were below 95% predictive limits derived from body surface area (BSA) adjusted normal data. PVCs were detected in 3 of these 4 patients, but were not found in any patient with heart size appropriate for BSA. Repeat echos in 6/9 patients after 27% mean weight gain, 1-6 months after initial study, showed increased Dd (13%), LV mass (28%) and left atrial dimension (21%) in all 6 patients; reduced dimensions became normal in each instance. We conclude that cardiac size may increase rapidly with weight gain in Anorexia Nervosa. Ventricular ectopy may be present, despite normal LV mechanical function, particularly in patients in whom heart size corrected for BSA is low.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01692-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Cardiac Effects of High Dose Cyclophosphamide

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

PI:	J. S. Gottdiener	Expert	CB NHLBI
Other:	F. Appelbaum	Clinical Investigator	POB - NCI
	A. B. Deisseroth	Head, Experimental Hematol. Sec.	POB - NCI

COOPERATING UNITS (if any)

Pediatric Oncology Branch, NCI (NIH)

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.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)

The cardiac effects of high dose (180 mg/kg) cyclophosphamide (CTX) were studied in 14 patients by serial echocardiograms and ECG. The ECG's of an additional 17 patients were reviewed. Summated QRS voltage (Σv) decreased in 27/31 (81%) patients with maximum decline 2-14 days (mean 7 days) after beginning CTX. Fractional shortening decreased in 10/13 patients (77%). Although pericardial effusion occurred in 9/31 patients (29%); CTX was associated in with decreased Σv in 8 patients in whom effusion was excluded by echo. Congestive heart failure occurred in 12/31 (39%) patients resulting in 7 deaths (23%) 6-14 (mean 10 days) after starting CTX. Morbidity and mortality from cardiac toxicity of this regimen appear to be high.

546

PHS-6040
(Rev. 10-76)

Project Description: To determine the cardiac effects of high dose cyclophosphamide (CTX), serial echocardiograms (echo) and electrocardiograms (ECG) were obtained in 14 patients receiving CTX 45 mg/kg for 4 days (total 180 mg/kg). ECGs of an additional 17 patients were examined. Summated QRS voltage in 1, AVF and VI (ΣV) decreased in 27/31 (87%) patients with maximum decline from s.3 $\pm$ 0.3 to 1.9 $\pm$ 0.3 mv ($p<.005$) at 2-14 days (mean 7 days) after beginning CTX. Fractional shortening by echo decreased in 10/13 (77%) patients from .39 $\pm$ .04 to .30 $\pm$ .09 ($p<.01$). In 15 surviving patients mean ΣV (3.0 $\pm$ 1.3 mv) at >31 days after CTX was unchanged from pre-drug value of 3.3 $\pm$ 1.2 mv (pNS). Pericardial effusion (PE) occurred in 9/31 (29%) patients; 6 of these had tamponade. However, CTX was also associated with decreased ΣV (3.3 $\pm$ 1.1 to 2.2 $\pm$ 1.3 mv; $p<.005$) in 8 patients in whom PE was excluded by echo. Congestive heart failure (CHF) occurred in 12/31 (39%) patients, resulting in 7 deaths (23%) 6-14 (mean 10) days after starting CTX. We conclude that decreased ΣV is a frequent and early manifestation of CTX cardiac effect. This may be associated with depressed left ventricular function and PE, but may occur independently of either. Morbidity and mortality from cardiac toxicity of this regimen are high. However, functional and electrocardiographic recovery in surviving patients appears to be complete.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01693-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Long-term Fate of Coronary Artery Bypass Grafts: Anatomic and Functional Status at Five Years

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

PI:	S. F. Seides	Senior Investigator	CB NHLBI
Other:	J. S. Borer	Senior Investigator	CB NHLBI
	K. M. Kent	Head,Sec.on Cardiovasc.Diag.	CB NHLBI
	D. R. Rosing	Senior Investigator	CB NHLBI
	C. L. McIntosh	Senior Surgeon	SB NHLBI
	S. 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 20014

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)

Twenty patients who underwent coronary artery bypass grafting prior to 1973 were studied. In these 20 patients (1) 28/30 (93%) of grafts that were patent several months post-op remained so for at least 5 years and (2) late symptomatic deterioration was common, most often due to progression of disease in ungrafted vessels.

548

PHS-6040
(Rev. 10-76)

Project Description: Thirty consecutive patients having coronary artery bypass grafts (CABG) prior to 1973 had at least one graft patent at an early (3-9 mos) post-op study. Of these, 20 pts (with 30 patent grafts) were available for follow-up evaluation. Coronary and graft angiography were performed 53-84 mos (mean 66 mos) post-op. Of the 30 grafts studied, 28 (93%) were patent at late study. At least one graft was patent in 19/20 patients. Despite this, symptomatic deterioration was common. All 20 patients preoperatively were Functional Class (FC) 3 or 4. Early post-operatively 16 became FC 1. Of these, 11/16 (69%) deteriorated at least one FC by late study. Progression of disease in ungrafted vessels accounted for symptomatic deterioration in 7 of these 11 patients with only 1/11 demonstrating graft occlusion. No patient had progression of disease distal to a patent graft nor did any patient develop disease in a previously normal vessel.

We conclude that 1) most grafts that are patent several months postoperatively remain so for at least 5 years and 2) although most patients improve symptomatically following operation, symptomatic deterioration is common in the succeeding years and this most often is due to progression of disease in ungrafted vessels.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01694-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Effect of Pacing on Hemodynamics in Patients with Hypertrophic Cardiomyopathy (ASH)

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

PI:	S. F. Seides	Senior Investigator	CB	NHLBI
Other:	K. M. Kent	Head, Sec. on Cardiovasc. Diag.	CB	NHLBI
	D. R. Rosing	Senior Investigator	CB	NHLBI
	J. S. Borer	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 20014

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)

Six patients with ASH have been studied with incremental atrial and ventricular pacing during diagnostic cardiac catheterization. The data suggest that (1) the detrimental clinical effect of atrial or ventricular tachyarrhythmias in primarily mediated by an elevation in filling pressure and a decrease in cardiac output rather than augmentation of outflow obstruction and (2) at slow rates (≤110 bpm) atrial pacing is hemodynamically advantageous.

550

PHS-6040
(Rev. 10-76)

Project Description: Patients with ASH often experience profound symptomatic deterioration with onset of rapid atrial or ventricular tachyarrhythmias. It is not known whether such deterioration is due to increase in gradient or to inadequate cardiac filling.

To assess the effects of heart rate and pacing site on hemodynamics in patients with hypertrophic cardiomyopathy six patients undergoing diagnostic cardiac catheterization have been studied. All patients were symptomatic; two of six patients had significant resting left ventricular outflow obstruction ($\leq$50 mmHg); the remaining four patients developed significant left ventricular outflow obstruction with provocative maneuvers. Left ventricular, brachial artery and pulmonary capillary wedge pressures and cardiac output were determined at rest and during incremental right atrial and right ventricular pacing. Maximum heart rate averaged 168 beats/min during atrial pacing and 155 beats/min during ventricular pacing. Both patients with resting left ventricular outflow tract obstruction had a decrease in outflow tract gradient with atrial and ventricular pacing and none of four patients with provocable obstruction developed an outflow tract gradient during pacing. At maximal rates pulmonary capillary wedge pressure rose an average of 120% in five of six patients with atrial pacing and an average of 94% with ventricular pacing in six of six patients. Cardiac output decreased an average of 28% in four of six patients during atrial pacing and decreased an average of 34% in four of six patients with ventricular pacing. At slower rates (110 beats/min) atrial pacing caused no consistent changes in pulmonary capillary wedge pressure or in cardiac output while ventricular pacing caused an increase in pulmonary capillary wedge pressure in 5 of 6 patients and a decrease in cardiac output in 3 of 4 patients.

These data would seem to imply that (1) the detrimental hemodynamic effect of rapid atrial and ventricular tachyarrhythmias in hypertrophic cardiomyopathy are mediated by an increase in left ventricular filling pressure and a decrease in cardiac output rather than augmentation of left ventricular outflow obstruction and (2) at lower heart rates atrial pacing is considerably more hemodynamically advantageous than ventricular pacing in managing patients with hypertrophic cardiomyopathy.

Publications: None

(2)
(3)

END

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01695-01 CB

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Effect of Drugs on the Pulmonary Circulation in Patients with Primary Pulmonary Hypertension

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

PI:	S. F. Seides	Senior Investigator	CB NHLBI
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	D. R. Rosing	Senior Investigator	CB NHLBI
	J. S. Borer	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 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.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)

Three patients with primary pulmonary hypertension have been studied in the basal state and after administration of nitroprusside, nitroglycerin, isoproterenol and tolazoline. One patient had a marked decrease in total pulmonary resistance with all drugs. The response of the pulmonary vascular bed to drugs in these patients appears unpredictable and in particular, isoproterenol may be hazardous in some patients by increasing cardiac output and pulmonary pressure.

552

PHS-6040
(Rev. 10-76)

To study the effect of various potential pulmonary vasodilators in patients with primary pulmonary hypertension three patients have been studied in the basal state and during administration of nitroprusside, nitroglycerin, isoproperenol and tolazoline. One patient had a dramatic response to all drugs with a 64% decrease in total pulmonary resistance during isoproterenol infusion and a 59% decrease in total pulmonary resistance after tolazoline administration. The remaining two patients had only a small ($\leq$20%) change in total pulmonary resistance with any drug. In these two patients, isoproterenol caused a 55% and a 17% increase in mean pulmonary artery pressure along with a corresponding increase in cardiac output. These data would seem to indicate that (1) the response of the pulmonary vascular bed to vasodilators in patients with primary pulmonary hypertension is unpredictable, and an individualized drug trial prior to therapy is mandatory, and (2) isoproterenol administration may be hazardous in some patients by causing a major increase in pulmonary arterial pressure due to augmentation of cardiac output with a fixed pulmonary resistance.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01696-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Echocardiographic evaluation of patients with essential hypertension

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

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	W. L. Henry	Senior Investigator	CB NHLBI
	E. C. Mathews, Jr.	Guest Worker	CB NHLBI
	S. E. Epstein	Chief, Cardiology Branch	CB NHLBI
	J. H. Laragh	Master Professor	N.Y.Hosp.-Cornell Med.Center

COOPERATING UNITS (if any)
Cardiovascular Center, New York Hospital - Cornell Medical Center

LAB/BRANCH
Cardiology Branch

Section
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI- NIH, Bethesda, Maryland 20014

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)

Patients with essential hypertension were studied by echocardiography to assess the prevalence of cardiac abnormalities in these patients and to compare this method with chest roentgenography and electrocardiography for detecting these abnormalities.

554

Project Description: To characterize the prevalence of cardiac abnormalities, including evidence of primary or secondary cardiomyopathies, in patients with essential hypertension (untreated BP>140/90) 262 patients (mean age =48; range 15-75) were studied by echocardiography (echo). Of these, 109 patients were untreated. Concentric hypertrophy ($\geq$1.3 cm) of the ventricular septum (VS) and left ventricular posterior wall (LVPW) was found in 47% (109/233). Increased LV internal dimension at end-diastole (LVIDd>5.2cm) was found in 13% (27/204). Ejection fraction was decreased ($\leq$60%) in 8% (16/214), 75% (12/16) of whom had concentric LV hypertrophy. Only 1 patient clearly had a dilated cardiomyopathy with an ejection fraction of 23% and LVIDd=6.0 cm. Asymmetric septal hypertrophy (ASH) (VS/LVPW $\geq$1.3) was found in 4% (10/233). Increased left atrial dimension (LA>4.0 cm) was found in 13% (27/204). Three patients had pericardial effusions and 2 had mitral valve prolapse. These data suggest a high prevalence of cardiac abnormalities in hypertensive patients, most of whom were asymptomatic, had normal ECG's and normal chest radiographs. Dilated cardiomyopathy is less common than ASH in this population.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01697-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Pre- and Postoperative Evaluation of Ventricular Function in Aortic Regurgitation

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

PI:	R. O. Bonow	Clinical Associate	CB	NHLBI
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	A. S. Pearlman	Clinical Associate	CB	NHLBI
	D. D. Savage	Clinical Associate	CB	NHLBI
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	J. S. Borer	Senior Investigator	CB	NHLBI
	C. L. McIntosh	Senior Surgeon	SB	NHLBI
	A. G. Morrow	Chief, Surgical Branch	SB	NHLBI
	S. E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

Surgical Branch, NHLBI

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1.5	.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)

Thirty-one patients with aortic regurgitation (AR) were evaluated pre- and postoperatively by electrocardiography, echocardiography, cardiac catheterization, and graded treadmill exercise testing. Exercise testing was found to be an inaccurate method for identifying left ventricular dysfunction in patients with AR and was not predictive of early or late postoperative mortality. Preliminary echo data suggest that patients with preop ejection fractions $\leq$ 64% have a high incidence of early and late postop mortality.

556

Project Description: Correct timing of operation for acortic regurgitation (AR) before irreversible myocardial damage occurs is a difficult problem. As part of the AR protocol, we are evaluating left ventricular (LV) function pre- and postoperatively by electrocardiography, echocardiography, cardiac catheterization, and graded treadmill exercise (ex) testing to determine preoperative parameters that would be predictive of poor postop responses. Total ex time, maximum (max) heart rate, and max O_2 consumption (VO_2) were used to evaluate ex performance.

Patients with echo ejection fractions ≤64% exercised longer (mean 17.7 min) than those with ejection fractions >64% (mean 10.4 min, $p<.05$). This occurred despite higher LV end-diastolic pressures and lower forward cardiac indices in the patients with the lower ejection fractions. Increased ex performance was also associated with higher echo LV diastolic dimensions and mass and larger angiographic LV diastolic volumes. Max heart rate and max VO_2 were similar in the two groups. Exercise capacity did not correlate with severity of symptoms or early or late postop mortality. Thus, exercise testing is not an accurate method for identifying LV dysfunction in patients with AR and clinically may give a false sense of security in a patient with a severely compromised ventricle.

Preliminary echo data suggest that patients with ejection fractions ≤64% preop have a high incidence of early (immediate to 6 months postop) and late (over 6 months) postop mortality; ejection fractions ≤64% at 6 months postop are also predictive of higher likelihood of late postop mortality:

	Preop Echo					6-month Postop Echo		
	Postop mortality							
	Pts	early	late	total	total%	Pts	Late Mortality	%
Ejection fraction ≤64%	17	3	6	9	52.9	13	6	46.0
Ejection fraction >64%	14	0	2*	2	14.2	8	1	12.5

* One of these two patients experienced an intraoperative myocardial infarction and had an ejection fraction <64% postoperatively.

Although there was no correlation between preoperative LV diastolic dimension (LVDD), large LVDD (>65mm) 6 months postop correlated with late mortality:

	6-month Postop Echo		
	Pts	late mortality	%
LVDD ≤ 65mm	15	3	20.0
LVDD > 65mm	6	4	66.7

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (DO NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01698-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Hemodynamic Response to Intense Upright Exercise Following Myotomy and Myectomy in Patients with ASH

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

PI:	D. R. Redwood	Head, Sec. on Cardiovasc. Diagnosis	CB	NHLBI
Other:	R. E. Goldstein	Senior Investigator	CB	NHLBI
	J. Hirshfeld	Clinical Associate	CB	NHLBI
	J. Morganroth	Clinical Associate	CB	NHLBI
	A. G. Morrow	Chief, Surgical Branch	SB	NHLBI
	S. E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COORDINATING UNITS (if any)

Surgical Branch, NHLBI

LAB / BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1	.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Myotomy and myectomy results in marked symptomatic improvement in the majority of patients with ASH. However, the effect of operation on exercise capacity and cardiac function remains controversial. We found operation markedly improves cardiac function during exercise.

PHS-6040
(Rev. 10-76)

Project Description: Myotomy and myectomy results in marked symptomatic improvement in most patients with ASH. However, the effect of operation on exercise capacity and cardiac function remains controversial. Accordingly, 29 patients were evaluated during graded treadmill exercise. Postoperatively all patients were symptomatically improved and had marked reduction or abolition of left ventricular outflow gradients. Significant increases occurred in exercise duration (7.2 to 15.7 min, $p<.001$) and peak VO_2 (16.2 to 21.3 ml/min/kg, $p<.001$). A significant increase in cardiac index during maximal exercise accompanied this improved performance (5.03 to 5.63 $l/min/m^2$, $p<.05$). The increase in maximal index was associated with an increase in maximal desaturation of mixed venous blood (29.2 to 22.8%, $p<.01$). At a given level of venous desaturation(30%), cardiac index was higher opstoperatively (4.55 to 5.21 $l/min/m^2$, $p<.05$). These results suggest that while several mechanisms may contribute to symptomatic improvement following operation (including placebo effect, increased O_2 extraction and training) enhanced cardiac performance plays an important role in most patients. Moreover, these results demonstrate that abolition of the gradient following operation is not caused by surgically induced myocardial damage.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01699-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Effects of Nitroglycerin on Myocardial Blood Flow in Exercising Dogs with Coronary Disease

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

PI:	K. Marr	Clinical Associate	RO	NCI
Other:	W. H. Heaton	Clinical Associate	CB	NHLBI
	N. L. Capurro	Sr. Staff Fellow	CB	NHLBI
	R. E. Goldstein	Senior Investigator	CB	NHLBI
	S. E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)
Radiation Oncology Branch, National Cancer Institute

LAB/BRANCH
Cardiology Branch

Section
Experimental Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.6	.5	.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)

To measure the effects of nitroglycerin during exercise on animals with coronary occlusive lesions, we used radioactive microspheres to assess coronary blood flow at rest and during exercise before and after nitroglycerin in dogs with surgically induced obstructive coronary disease. One region of myocardium, the endocardial portion of the zone perfused exclusively by collateral channels, showed evidence of deficient blood supply. This region had either no change or possibly a slight increase in coronary blood flow during exercise after nitroglycerin despite a fall in perfusion pressure. Thus our data suggest a nitroglycerin-induced salutary influence on coronary collateral function during exercise.

560

Project Description: Nitroglycerin increases regional myocardial blood flow during acute coronary occlusion in dogs with otherwise normal coronary arteries but its effect during exercise in the presence of pre-existing multivessel coronary disease is unknown. We therefore measured hemodynamic indexes and myocardial blood flow with radioactive microspheres (15μ) at rest and during exercise, before and after nitroglycerin in 12 dogs with pre-existing two vessel coronary occlusive disease. The left anterior descending coronary artery was gradually occluded by an ameroid constrictor while the left circumflex coronary artery was restricted 60 to 90% by a partially occlusive ligature.

Two to four weeks later, exercise raised transmural myocardial blood flow 56% in the normal zone and 56% in the collateral dependent zone adjacent to the left anterior descending ($p<.01$). Initially mean endocardial myocardial blood flow in collateral dependent zone was $1.04 \pm .11$ ml/min/g or 85% of normal zone at rest ($p<.05$) and $1.54 \pm .21$ ml/min/g or 82% of normal zone during exercise ($p<.01$), indicating relative hypoperfusion. During intravenous TNG (390 ± 70 mcg/min), blood pressure decreased 117 to 94 ($p<.001$) at rest, and 122 to 108 ($p<.01$) during exercise with no significant heart rate change. Nitroglycerin increased transmural (37%, $p<.01$) and epicardial (43%, $p<.01$) flow during exercise in the collateral dependent zone with no change in endocardial flow. Nitroglycerin produced no significant changes in collateral dependent zone flow at rest.

Thus, nitroglycerin during exercise raised flow significantly to all regions except the hypoperfused endocardium. However, maintenance of endocardial flow despite blood pressure decrease may represent an important benefit of nitroglycerin.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01700-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Effect of Propranolol Withdrawal on Sympathetic Function and on Platelets of Normal Humans

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

PI:	R. E. Goldstein	Senior Investigator	CB NHLBI
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	C. R. Lake	Staff Psychiatrist	Lab.Clin.Science-DCBR,NIMH
	H. R. Keiser	Chief, Exper.Therap. Sec.	HE NHLBI
	N. L. Capurro	Sr. Staff Fellow	CB NHLBI
	S. E. Epstein	Chief, Cardiology Branch	CB NHLBI

COOPERATING UNITS (if any)
Hypertension-Endocrine Branch, CC, NIH
Hematology Service, CC, NIH
Experimental Therapeutic Sec., NIH

LAB/BRANCH
Cardiology Branch

Section
Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLBI- NIH, Betheda, Maryland 20014

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

To evaluate the possibility that abrupt withdrawal of propranolol causes overactivity of the sympathetic nervous system or enhancement of platelet function, we examined sympathetically-mediated heart rate responses, platelet survival and aggregation, and plasma and urine catecholamines in 10 normal subjects before and at various times after propranolol therapy. Preliminary results suggest that propranolol withdrawal may be associated with shortened platelet survival. No consistent change was noted in heart rate responses to direct and reflex sympathetic stimulation.

562

PHS-6040
(Rev. 10-76)

Projects Description: Abrupt withdrawal of propranolol therapy in patients with coronary artery disease has been associated with worsening of ischemic symptoms relative to pre-treatment status and with acute myocardial infarction. Since proporanolol acts by blocking the beta-adrenergic receptor and may also inhibit the function of platelets, we examined the influence of abrupt withdrawal of propranolol upon adrenergically mediated heart rate responses and upon platelet survival. Ten normal volunteers had baseline measurement of heart rate response to upright tilt (before and after atropine), exercise and isoproterenol infusion. Baseline platelet survival was measured by a chromium labelling technique. Subjects were then given propranolol 80 to 240 mg daily for 3 to 10 weeks. Physiologic measurements were repeated on the first, second and eighth days after abrupt withdrawal and a second determination of platelet survival was made in the withdrawal period. Preliminary results suggests that there is no consistent increase in heart rate responses to direct stimulation by beta-adrenergic agonist by exercise or by reflex mechanisms during the withdrawal period. Data from the first five patients, however, suggests that there may be a consistent shortening of platelet survival times. It should be stressed that evaluation of observations is still in progress and that final judgement should be reserved with regard to the conclusions to be drawn from measurements made in our ten subjects. In addition, blood was obtained to determine catecholamine levels at rest, during upright posture, and during upright exercise in six subjects prior to, during, and after treatment with propranolol. Blood was also obtained for quantitative assessment of leukocyte beta-adrenergic receptors before, during, and after propranolol. Results of these tests plus determination of serum propranolol levels are still pending.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01701-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Radionuclide Cineangiography and Chest Pain Without Coronary Artery Disease

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

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	M. V. Green	Chief, Applied Physics Sec.	NM	CC
	K. M. Kent	Head, Sec. on Cardiovasc. Diagnosis	CB	NHLBI
	D. R. Rosing	Senior Investigator	CB	NHLBI
	S. F. Seides	Senior Investigator	CB	NHLBI
	G. S. Johnston	Chief, Nuclear Medicine Dept.	NM	CC
	S. E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)
Nuclear Medicine Department, CC.

LAB/BRANCH
Cardiology Branch

Section
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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)

Many patients who present with chest pain do not have coronary artery disease, though this cannot be determined without invasive catheterization studies. Radionuclide cineangiography provides an accurate means of assessing the presence of coronary artery disease on patients with CAD. Also the technique appears to be useful both in ruling out ischemic heart disease in those without CAD, and in demonstrating global dysfunction, suggestive of some sort of cardiomyopathic process, in some of these patients. Therefore, we use radionuclide cineangiography in the assessment of left ventricular function at rest and during exercise in all patients with chest pain suggesting CAD, including those patients with mitral valve prolapse, asymmetric septal hypertrophy and aortic stenosis as well as those with chest pain without other apparent cause.

564

PHS-6040
(Rev. 10-76)

Project Description: Many patients who present with chest pain do not have coronary artery disease, though this cannot be determined without invasive catheterization studies. Radionuclide cineangiography provides an accurate means of assessing the presence of coronary artery disease on patients with CAD. Also the technique appears to be useful both in ruling out ischemic heart disease in those without CAD, and in demonstrating global dysfunction, suggestive of some sort of cardiomyopathic process in some of these patients. Therefore, we use radionuclide cineangiography in the assessment of left ventricular function at rest and during exercise in all patients with chest pain suggesting CAD, including those patients with mitral valve prolapse, asymmetric septal hypertrophy and aortic stenosis as well as those with chest pain without other apparent cause. Thus far 90 of these patients have been free of CAD, and have provided data for the analysis of the specificity of radionuclide cineangiography in the diagnosis of coronary artery disease. Of these 90 patients, all those with aortic stenosis (30 patients) have had reduction in global ejection fraction with exercise, but one demonstrated regional dysfunction suggestive of CAD. Patients with asymmetric septal hypertrophy (30 patients) have increased or decreased ejection fraction with exercise, but have not developed regional dysfunction, suggesting that their pain may not be related to ischemia of any large area of myocardium and may not be associated with ventricular dysfunction. Five of the 15 patients with mitral valve prolapse have demonstrated reduction in global ejection fraction with exercise, one with wall motion abnormalities, despite the absence of coronary artery disease. None of the 18 patients with chest pain of unknown etiology without coronary artery disease have developed regional dysfunction though two have shown global dysfunction with exercise. Thus radionuclide cineangiography is highly specific for CAD in patients without valvular disease, but may demonstrate regional, as well as global dysfunction, in patients with AS or mitral valve prolapse. The findings in patients with MV prolapse are consistent with the presence of a cardiomyopathic process in these patients.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01702-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Radionuclide Cineangiographic Assessment of Patients with Aortic Regurgitation

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

PI:	J. S. Borer	Senior Investigator	CB	NHLBI
Other:	S. L. Bacharach	Physicist	NM	CC
	M. V. Green	Chief, Applied Physics Sec.	NM	CC
	K. M. Kent	Head, Sec. on Cardiovasc. Diag.	CB	NHLBI
	S. E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)
Nuclear Medicine Department, CC, NIH.

LAB/BRANCH
Cardiology Branch

Section
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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)

Radionuclide cineangiography during exercise permits accurate characterization of the functional reserve of the left ventricle. We have used the technique to evaluate our patients with aortic regurgitation. Abnormalities were noted not only in all of the symptomatic patients, but in many totally asymptomatic patients as well.

Project Description: The usual indication for operation in patients with aortic regurgitation (AR) is onset of symptoms. Although this approach avoids premature operation, about 50% of patients operated on for this indication are dead within 4 years after operation. Indirect evidence suggests that most patients in this subgroup had poor left ventricular function (LVF) preop; symptoms often appeared only after LVF had markedly deteriorated. Hence, a method is needed to detect earliest evidence of impaired LVF. We have developed a real-time movie display technique for scintigraphic-count-based analysis of LVF during exercise using gated Tc^{99m} radionuclide angiograms. With this technique, we have assessed LVF in 12 patients with hemodynamically severe AR and 14 age-matched normal subjects (N) at rest and during supine bicycle exercise. Six AR patients were asymptomatic. Ejection fraction (EF) increased during exercise in all N (avg = 50 $\pm$ 4 (S.D.)% at rest, 69 $\pm$ 1% during exercise; $p<.001$), but decreased in 9 of the 12 AR patients (mean decrement 22%, range = 11 to 34%). EF decreased in all 6 AR patients with any symptoms (minimal to severe), and also in 3 of 6 asymptomatic patients. EF was normal at rest in 9 patients; in 6 of these EF decreased with exercise. We conclude that in patients with AR (1) exercise-induced decrease in LVF precedes the development of symptoms in many (and perhaps most) patients; (2) LVF becomes abnormal with exercise before changes occur at rest. Since abnormal LVF at rest is associated with poor long-term survival after operation, periodic radionuclide assessment of LVF during exercise may be of unique value in determining optimal timing of operation in patients with AR.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01703-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Left Ventricular Function During Exercise Before and After Aortic Valve Replacement

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

PI:	J. S. Borer	Senior Investigator	CB	NHLBI
Other:	S. L. Bacharach	Physicist	NM	CC
	M. V. Green	Chief, Applied Physics Sec.	NM	CC
	K. M. Kent	Head, Sec. on Cardiovasc. Diagnosis	CB	NHLBI
	D. R. Rosing	Senior Investigator	CB	NHLBI
	S. F. Seides	Senior Investigator	CB	NHLBI
	C. L. McIntosh	Senior Surgeon	SB	NHLBI
	D. M. Conkle	Senior Surgeon	SB	NHLBI
	A. G. Morrow	Chief, Surgery Branch	SB	NHLBI
	S. E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

Surgery Branch, NHLBI
Nuclear Medicine Department, CC, NIH.

LAB/BRANCH
Cardiology Branch

Section
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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)

Radionuclide cineangiography during exercise allows accurate characterization of the functional reserve of the left ventricle. We have used the technique routinely to evaluate the status of patients with aortic regurgitation after aortic valve replacement, and have compared these results with the findings in the same patients before operation. While [illegible] markedly after operation, it does not return to normal

Project Description: Using non-invasive radionuclide cineangiography we have previously shown in all symptomatic patients and many asymptomatic patients with aortic regurgitation (AR) that during exercise ejection fraction (EF) falls as compared with resting values; moreover, this occurs before ECG or resting left ventricular end-diastolic pressure abnormalities develop. Normal subjects (35) always increase EF (average increase 25%, $p<.001$) with exercise. To determine the effect of aortic valve replacement (AVR) in reversing this abnormality in left ventricular function (LVF), we studied 8 patients with AR pre- and 6 months post-AVR. Pre-op EF was normal (average 50%) at rest in all patients. However, EF invariably fell with exercise (average EF = 40%), $p<.01$). After AVR, EF at rest (average 56%) was not significantly changed from pre-op; EF during exercise fell to 53%, higher than the pre-op value ($p<.025$) but lower than EF during exercise in normal subjects (average 71%, $p<.001$). We conclude that AVR can improve, but does not normalize, LVF during exercise in symptomatic patients with AR. Long-term survival is poor after AVR in these patients. Evaluation of LVF during exercise may provide a better indicator to judge optimal time for operation in patients with AR than symptoms or other indices now used.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01704-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Comparison of Radionuclide Cineangiography and Exercise Electrocardiography

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

PI:	J. S. Borer	Senior Investigator	CB	NHLBI
Other:	K. M. Kent	Head, Sec. on Cardiovasc. Diagnosis	CB	NHLBI
	S. L. Bacharach	Physicist	NM	CC
	M [illegible] Green	Chief, Applied Physics Sec.	NM	CC
	[illegible]	Chief, Cardiology Branch	CB	NHLBI
	[illegible]	Chief, Nuclear Medicine Dept.	NM	CC

[illegible] Maryland 20014

OTHER:
.5

[illegible] HUMAN TISSUES [illegible] (c) NEITHER

[illegible] sensitive method to detect ischemic heart [illegible] we have compared the sensitivity of [illegible] exercise electrocardio- [illegible] are significantly more sensitive.

Project Description: Non-invasive radionuclide cineangiography (RC) permits assessment of global and regional left ventricular function during intense exercise. To assess the accuracy of RC we studied 50 consecutive patients with only CAD (≥50% stenosis of at least one coronary artery), including 13 who were asymptomatic during exercise and 15 with normal regional function at rest. Each demonstrated at least one new region of dysfunction with exercise. The last 40 of these patients also underwent exercise electrocardiography (upright bycycle) to angina or to 85% predicated maximal heart rate. Significantly fewer (19 of 40) developed an abnormality indicative of CAD (≥1 mm ST segment depression), as compared to RC (40 of 40, $p<.01$). To assess specificity of RC we studied 16 patients with chest pain but with normal coronary arteries. There were no false positive RC results. In addition, we studied 4 asymptomatic patients with positive exercise ECGs but with normal coronary arteries. None had RC abnormalities. We conclude that RC is a highly sensitive indicator of CAD. Moreover, its sensitivity is considerably superior to that of ST response to exercise. Although more patients must be studied, these preliminary data suggest RC also has high specificity and predictive accuracy.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space

PERIOD COVERED
July 1, 1976 to September 30

Project Description: Twenty patients with ASH, with or without obstruction, and history of presyncope/syncope and/or family history of sudden cardiac death have been studied by His Bundle electrography to assess possible conduction abnormalities which might lead to symptoms or death. Thus far, five have demonstrated prolongation of H-V time, indicating disease of the conduction system distal to the AV node and known to be associated with a high incidence of atrioventricular block with low escape heart rates. Another six have manifested re-entry rhythms during premature atrial stimulation, suggesting a proclivity for the development of supraventricular tachycardias which, by causing a sudden and pronounced fall in arterial pressure in patients with ASH, might lead to syncope, and perhaps to myocardial ischemia with further, potentially lethal, arrhythmias. No patient has demonstrated evidence of a bypass tract.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01706-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Prognostic Valve of Non-invasive Assessment after Acute Myocardial Infarction

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

PI:	J. S. Borer	Senior Investigator	CB NHLBI
Other:	K. M. Kent	Head, Sec. on Cardiovasc. Diagnosis	CB NHLBI
	D. R. Rosing	Senior Investigator	CB NHLBI
	S. F. Seides	Senior Investigator	CB NHLBI
	S. L. Bacharach	Physicist	NM CC
	M. V. Green	Chief, Applied Physics Sec.	NM CC
	D. Holmes	Physician	Nat. Naval Med. Center
	H. Cohen	Physician	" " " "
	W. Baker	Physician	" " " "
	D. Donohue	Physician	Walter Reed Army Med.Ctr.
	J. Bedynek	Physician	" " " " "
	S. E. Epstein	Chief, Cardiology Branch	CB NHLBI

COOPERATING UNITS (if any)
Nuclear Medicine Department- CC- NIH
National Naval Medical Center-Bethesda, Md.
Walter Reed Army Medical Center -Washington, D.C.

LAB/BRANCH
Cardiology Branch

Section
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.3	.25	.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)

Left ventricular function at rest and arrhythmia analysis prior to hospital discharge allows prediction of a portion of the 10-15% of patients who will die suddenly within six months of discharge after acute myocardial infarction. Using new non-invasive radionuclide cineangiographic techniques which permit study during exercise and hence provide more complete assessment of cardiac function than previously available, we propose to determine the feasibility of improving prognostic capability after acute MI. Better prediction might allow appropriate application of medical and surgical therapy prophylactically in high risk patients.

574

PHS-6040
(Rev. 10-76)

Project Description: Left ventricular function at rest and arrhythmia analysis prior to hospital discharge allows prediction of a portion of the 10-15% of patients who will die suddenly within six months of discharge after acute myocardial infarction. Using new non-invasive radionuclide cineangiographic techniques which permit study during exercise and hence provide more complete assessment of cardiac function than previously available, we propose to determine the feasibility of improving prognostic capability after acute myocardial infarction. Better prediction might allow more appropriate application of medical and surgical therapy prophylactically in high risk patients.

One day prior to hospital discharge in survivors of acute myocardial infarction, and after tapering cardiac medications (when possible), we record heart rhythm for 24 hours on a magnetic tape which is analyzed for rhythm disturbances. Patients then undergo moderate exercise stress testing for arrhythmia analysis, and for determination of left ventricular function by radionuclide cineangiography. Patients are then discharged from the hospital and are to be followed for one year, to determine subsequent mortality and morbidity. Six months after discharge, the arrhythmia and radionuclide assessments are to be repeated. Thus far, two patients have received their initial assessment. Neither manifested arrhythmia with exercise, though one had frequent PVCs on 24 hour monitoring. Both had only mildly depressed left ventricular function with exercise, and both are alive one month after assessment.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01707-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Effect of Nitroglycerin on Global and Regional Left Ventricular Function

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

PI:	J. S. Borer	Senior Investigator	CB NHLBI
Other:	S. L. Bacharach	Physicist	NM CC
	M. V. Green	Chief, Applied Physics Sec.	NM CC
	K. M. Kent	Head, Sec. on Cardiovasc.Diag.	CB NHLBI
	G. S. Johnston	Chief, Nuclear Med. Dept.	NM CC
	S. E. Epstein	Chief, Cardiology Branch	CB NHLBI

COOPERATING UNITS (if any)
Nuclear Medicine Department, CC, NIH.

LAB/BRANCH
Cardiology Branch

Section
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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)

Radionuclide cineangiography during exercise has been used to assess the functional severity of coronary artery disease and the effect of nitroglycerin on exercise-induced dysfunction in patients with and without symptoms. Even asymptomatic patients invariably reveal diagnostic abnormalities in regional and global left ventricular function during exercise, and nitroglycerin markedly mitigates these abnormalities.

576

Project Description: Nitroglycerin (TNG) prevents ischemic symptoms in patients with coronary artery disease (CAD) when administered before exercise. However, its effects on myocardial dysfunction provoked by exercise, particularly in the absence of symptoms, and hence its potential prophylactic role in such situations, is not known. Recently, we developed an ECG gated, radionuclide cineangiographic technique with which we have assessed global and regional left ventricular (LV) function at rest and during maximal supine bicycle exercise in 50 patients with CAD; 6 had no symptoms; 20 patients, including all 6 without symptoms, were restudied after TNG. Twenty six age matched normal subjects (Nl) also were studied. Whether or not regional dysfunction was present at rest, all CAD patients developed at least one new region of dysfunction during exercise, even in the absence of symptoms; LV ejection fraction (EF) invariably decreased during exercise (mean EF 34%) as compared with rest (mean EF 46%, $p<.001$). During exercise after TNG, regions of dysfunction previously provoked by exercise were absent or markedly diminished in 18 of 20 patients including all asymptomatic patients: EF during exercise was improved (mean 32% during exercise pre TNG, 47% during exercise post TNG, $p<.001$). In Nl, exercise did not cause regional dysfunction; EF invariably increased with exercise (mean 56% at rest, 69% with exercise, $p<.001$), and TNG did not alter EF during exercise. We conclude that 1) in patients with CAD, intense exercise invariably induces ischemic myocardial dysfunction even in the absence of symptoms; 2) TNG markedly ameliorates such ischemic dysfunction, but does not alter exercise performance of the normal LV. Therefore, chronic prophylactic therapy with nitrates may be advisable to prevent myocardial ischemia in CAD patients even during stresses which do not produce symptoms.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01708-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Real-time Radionuclide Cineangiography 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

PI:	J. S. Borer	Senior Investigator	CB	NHLBI
Other:	S. L. Bacharach	Physicist	NM	CC
	M. V. Green	Chief, Applied Physics Sec.	NM	CC
	K. M. Kent	Head, Sec. on Cardiovasc. Diag.	CB	NHLBI
	S. E. Epstein	Chief, Cardiology Branch	CB	NHLBI
	G. S. Johnston	Chief, Nuclear Medicine Dept.	NM	CC

COOPERATING UNITS (if any)
Dept. of Nuclear Medicine, CC, NIH.

LAB/BRANCH
Cardiology Branch

Section
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.2	.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)

Radionuclide cineangiography is a sensitive and accurate method for detecting the functional effects of coronary artery disease during exercise. Using the technique in patients with coronary disease, but normal left ventricular function at rest, we found diagnostic abnormalities during exercise in all patients.

PHS-6040
(Rev. 10-76)

Project Description:

Although coronary angiography defines regions of potential ischemia in patients with coronary artery disease, accurate assessment of the presence and functional importance of ischemia requires appraisal of regional and global left ventricular function during stress. To perform such assessment, we developed a noninvasive real-time radionuclide cineangiographic procedure permitting continuous monitoring and analysis of left ventricular function during exercise. In 11 patients with coronary disease who had normal regional and global ventricular function at rest, new regions of dysfunction developed during exercise ($P<0.001$), and in 10, global ejection fraction dropped 7 to 47 per cent. Fourteen age-matched normal subjects were studied; during exercise none had regional dysfunction, and each increased global ejection fraction (average increase, 23 ± 3 per cent [±S.E.], $P<0.001$ as compared with patients with coronary disease). Radionuclide cineangiography during exercise permits accurate assessment of the presence and functional severity of ischemic heart disease.

Publications:

Borer, J.S., Bacharach, S.L., Green, M.V., Kent, K.M., Epstein, S.E., Johnston, G.S.: Real-Time Radionuclide Cineangiography in the Noninvasive Evaluation of Global and Regional Left Ventricular Function at Rest and During Exercise in Patients with Coronary-Artery Disease. N. Engl. J. Med. 296: 839-844, 1977.

Project Description: Beta adrenergic blockade with propranolol can relieve angina pectoris, and may improve survival in some patients with coronary artery disease. However, because it reduces contractility, it may cause clinically important left ventricular dysfunction in some patients with coronary artery disease, and therefore is often withheld if evidence of left ventricular dysfunction exists in the absence of drug. However, it is possible that by reducing myocardial oxygen demand in such patients, propranolol might actually prevent ischemic dysfunction which otherwise might occur during stress. We are testing the efficacy of propranolol in this setting with the use of non-invasive radionuclide cineangiography, to determine whether the drug should be administered more freely to patients with coronary artery disease and left ventricular dysfunction.

After obtaining radionuclide movies of the heart with the patient at rest and during exercise, the patient is allowed to rest for 45 min and another study is performed at rest. Propranolol (.05 to 1 mg/kg) is administered intravenously over 5 to 10 minutes, and rest and exercise studies are repeated.

Thus far four patients have been studied. While reduction in global ejection fraction occurs at rest, no new wall motion abnormalities have been seen; no significant changes have yet been observed during exercise after drug administration in comparison with assessments performed in the absence of drug therapy.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01710-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Beneficial Effects of Physical Training on Collateral Blood Flow in the Exercising Dog

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

PI:	K. C. Marr	Clinical Associate	RO	NCI
Other:	W. H. Heaton	Clinical Associate	CB	NHLBI
	N. L. Capurro	Sr. Staff Fellow	CB	NHLBI
	R. E. Goldstein	Senior Investigator	CB	NHLBI
	S. E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNIT. (If any)

None

LAB/BRANCH
Cardiology Branch

Section
Experimental Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.2	.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)

To evaluate the influence of physical training on myocardial blood flow arriving via coronary collateral channels, we used radioactive microspheres to measure perfusion of the myocardium at rest and during exercise before and after physical training in dogs with multiple coronary occlusive lesions. The endocardial portion of the region of myocardium perfused occlusively by coronary collateral channels showed evidence of deficient flow, especially during exercise. Unlike other portions of the heart and in contrast to the myocardium of non-exercised control animals, these underperfused endocardial regions showed a substantial increase (40% rise) in blood flow during exercise after a 6-week program of treadmill exercise, suggesting an associated, training-induced improvement in collateral function.

Project Description: To determine the effect of physical training on myocardial blood flow delivered via chronic collaterals, 13 dogs were prepared with an ameroid constrictor about the left anterior descending coronary artery and a 70-90% fixed stenosis of the left circumflex. Two weeks later, regional myocardial blood flow was measured by radioactive microspheres at rest and during treadmill exercise. Six dogs performed exercise 5 days/week for 6 weeks while 7 remained in kennels. Myocardial blood flow studies were repeated at the end of 6 weeks. Samples were obtained from normally perfused zones and from regions distal to the occluded left anterior descending coronary artery (collateral dependent zones). Initially, endocardial myocardial blood flow at rest averaged 1.1 ml/min/g in collateral dependent zones (83% of normally perfused zones, $p<.05$) and during exercise averaged 1.4 (69% of normally perfused zones, $p<.05$), indicating the relative hypoperfusion of collateral dependent zones. After 6 weeks myocardial blood flow in collateral dependent zones during exercise was unchanged from initial exercise values in untrained dogs. However, endocardial myocardial blood flow during exercise in collateral dependent zones of trained dogs was 40% greater than prior to training ($p<.05$). The non-hypoperfused epicardial region of collateral dependent zones was unchanged after training. Thus our data suggest that the beneficial effects of physical training in coronary disease may include an improvement in myocardial blood flow during exercise to hypoperfused collateral dependent portions of myocardium.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HL 01711-01 CB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Phosphorylation of Myosin from Platelets, Macrophages and HeLa cells

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

PI:	J. A. Trotter	Guest Worker	CB	NHLBI
	B. Barylko	Visiting Fellow	CB	NHLBI
Other:	R. S. Adelstein	Head, Section on Molecular Cardiology	CB	NHLBI
	W. Anderson, Jr.	Chemist	CB	NHLBI
	J. M. Miles	Med. Biology Technician	CB	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Cardiology Branch

Section
Molecular Cardiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	.75	.25

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Myosin light chain kinase and phosphatase activity has been partially purified from rabbit macrophages and the kinase activity has been identified in HeLa cells. Myosin light chain phosphatase has been isolated from human blood platelets and partially characterized. This phosphatase dephosphorylates platelet myosin which results in a decrease in the actin-activated ATPase activity of platelet myosin.

584

PHS-6040
(Rev. 10-76)

Project Description:

The phosphorylating system of rabbit macrophages and HeLa cells: The enzyme myosin light chain kinase has been identified both in macrophages isolated from rabbit lungs and in HeLa cells. Moreover, mysoin light chain phosphatase activity has been found present in extracts of rabbit macrophage cells. The presence of the phosphorylating system in these two cells suggests that it may play a role in controlling actin-myosin interaction similar to the role played in other nonmuscle cells such as platelets and proliferative myoblasts. Using the phosphorylating system as a probe we hope to uncover some of the functions of actin and myosin in these two cells systems. In the macrophage system our attention will be directed to the role of the contractile proteins in cell motility and phagocytosis. In the case of HeLa cells we shall study the possible function of the contractile proteins in cell division.

Platelet myosin light chain phosphatase: Platelet myosin light chain phosphatase, the enzyme that catalyzes the dephosphorylation of phosphorylated platelet myosin has been partially purified. This enzyme has been shown to lower the actin-activated ATPase activity of platelet myosin concommitant with its dephosphorylation of the 20,000 dalton light chain of platelet myosin. That the only effect of the enzyme is to dephosphorylate the platelet myosin light chain was suggested by the following experiments: a) SDS-polyacrylamide gel electrophoresis of phosphorylated platelet myosin that was dephosphorylated with the phosphatase was unaltered after treatment with the enzyme. b) Platelet myosin that was dephosphorylated by the platelet phosphatase could be rephosphorylated using platelet myosin light chain kinase. c) Although the dephosphorylation of platelet myosin following treatment by the phosphatase resulted in a decrease in the actin-activated ATPase activity, the myosin ATPase activity measured in 0.5 M KCl in the presence of K^+-EDTA or calcium was unaltered. This shows that the myosin was not altered by dephosphorylation, but that the only effect of the enzyme was to reverse the effect of phosphorylation, i.e., was to decrease the actin-activated ATPase activity. The isolation of the phosphatase that dephosphorylates myosin shows that phosphrylation-dephosphorylation is a reversible regulatory mechanism in platelets.

ANNUAL REPORT OF THE
HYPERTENSION-ENDOCRINE BRANCH
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
July 1, 1976 through September 30, 1977

The work of the Hypertension-Endocrine Branch has included numerous studies in hypertensive patients with especial reference to renin, angiotensin, and sodium metabolism, studies in patients wtih Bartter's syndrome, in whom many of the vasoregulatory agents are abnormal, and clinical and laboratory studies on the control of renin secretion and of the action of prostaglandins on renal function. We have studied also all the aminergic neurotransmitters which may be related to the control of blood pressure, including dopamine, catechols, serotonin, and also the kallikrein-kinin system as related to blood pressure control.

Screening programs for hypertensive patients: In a study carried out in collaboration with the Section of Clinical Pharmacology, two hundred and forty-three hypertensive patients were evaluated first as outpatients, in an attempt to classify them according to a modification of the so-called renin-sodium-aldosterone index. The method proved of relatively little reliability as an outpatient procedure, correctly classifying only some two-thirds of the patients eventually considered to belong to the low-renin hypertension group. With the initiation of inpatient studies on these same patients, renin secretion was progressively stimulated by the use of diuretics and the use of diuretics combined with a low-sodium diet, taken for seven to fourteen days. By this method a small group of patients having low plasma renin activity despite all these maneuvers was identified for further study. The patients with low-renin hypertension, so defined, were studied for the response of plasma aldosterone to small, subpressor infusions of angiotensin II. By this means it was shown that the response in plasma aldosterone of the low-renin patients to infused angiotensin is significantly greater than that of normal subjects, or of hypertensive subjects with normal plasma renin activity. By this mechanism, the low-renin patients can maintain normal aldosterone secretion and excretion rates despite the very low plasma renin and plasma angiotensin II concentrations.

Aminoglutethimide as a therapeutic agent was studied in patients with low-renin hypertension. It was found that whereas aminoglutethimide lowered blood pressure in all subjects studied, and increased plasma renin into the normal range, it did not lower the concentration of desoxycorticosterone, 18-hydroxydesoxycorticosterone or of 16-beta-hydroxydehydroepiandrosterone, thus clearly dissociating the hypertensive state from the action of these steroids. The additional evidence thus provided renders the mediation of the hypertension by any one of these three steroids in low-renin hypertension most unlikely. Further, the view that any sodium-retaining steroid plays a part in this syndrome was not supported by studies of the activity of such a steroid in plasma to occupy aldosterone receptors in vitro in rat kidney slices. The plasma of patients with low-renin hypertension in this study showed no abnormal steroid capable of occupying these receptors by displacing labelled aldosterone therefrom.

Triamterene was studied as a blood pressure lowering agent in view of certain reports of its effectiveness. The drug was found to have relatively slight effect on blood pressure; in addition, it lowered creatinine clearance in a large proportion of the patients studied, and accordingly its use was discontinued.

Patients with "idiopathic" hypertension were studied for the sensitivity of blood pressure to dietary sodium. It was thus found that such patients fall into two groups: one showing significant increase in blood pressure in salt loading, and the other showing little or no increase. Plasma renin activity and plasma aldosterone concentration were the same in both groups, but the "salt-sensitive" patients retained significantly more of the administered sodium chloride, and gained significantly more weight than did the "non-salt-sensitive" patients. Current studies are underway to explain this phenomenon by exploration of catechol metabolism, of changes in cardiac output, and of differences in secretion of prostaglandins and bradykinin.

The relationship of prostaglandins to renin secretion was pursued at the clinical and the physiologic experimental level. The evidence that prostaglandins of the E series injected into the renal artery of dogs can increase renin secretion from the ipsilateral kidney was further confirmed and extended to the "non-filtering" kidney, wherein presumably a role for tubular prostaglandins or tubular sodium delivery is ruled out. This finding strengthens the impression that PGE can directly stimulate renin release from the juxtaglomerular cells, and conflicts with recent reports that PGE does not have such an effect in vitro in kidney slices.

The mediation of beta receptors in renin release was further studied by the use of propranolol in conscious and in salt-depleted dogs. Renin activity and plasma aldosterone concentration could be effectively lowered by propranolol, 5 mg/kg, followed by a sustained infusion. This supports the view that the hyperreninemia of the low-salt diet is in part mediated by beta stimulation. The role of prostaglandins in the mediation of water and sodium diuresis produced by bradykinin was measured in hypophysectomized dogs. It was found that bradykinin's ability to increase renal cortical blood flow is dependent upon prostaglandins, whose secretion is increased by bradykinin. Bradykinin, however, could still increase "delivery" of sodium and water to distal tubules, and thereby increase urinary sodium, in animals in which prostaglandin secretion had been markedly decreased with the use of indomethacin.

Further studies of the role of prostaglandins in renal function were done with the use of ACTH in normal volunteers. ACTH increased aldosterone secretion in normal volunteers, both before and after inhibition of prostaglandin synthesis by indomethacin. The effect was less with indomethacin, presumably because the plasma renin activity was significantly lower in the subjects receiving indomethacin. This provided further evidence that prostaglandins of the E series control renin secretion in part. The use of indomethacin with ACTH produced a considerable increase in urine solute concentration over that achieved with ACTH alone. This effect resulted presumably from a decrease of prostaglandins E production in the kidney: as prostaglandin E opposes the effect of vasopressin, the effect of its removal should increase the effectiven

ness of circulating vasopressin. Prostaglandin production by isolated renal medullary interstitial cells was explored *in extenso*. It was found that bradykinin, angiotensin II and arginine vasopressin stimulate the production of PGE in this preparation, presumably by the activation of phospholipase and thus by release of arachodonic acid; the action could be inhibited with the anti-lipase agent, mepacrine. In studies with the toad urinary bladder, it was found that vasopressin stimulated not only cyclic AMP production but also PGE biosynthesis. The PGE so produced inhibits the stimulation of water flow induced by the vasopressin. In these studies, it was also found that sulfonylureas act as prostaglandin synthetase inhibitors, thus enhancing vasopressin-stimulated water flow.

Studies in patients with Bartter's syndrome (in which plasma aldosterone and plasma renin activity are elevated and renal production of prostaglandin E is exaggerated) were continued with the help of inhibitors of prostaglandin synthetase. It was found that plasma bradykinin is elevated in the untreated patient, and decreases with the use of prostaglandin synthetase inhibitors, thus providing a partial explanation for the return of vascular sensitivity to angiotensin provided by these agents. The effect of PG synthetase inhibition to reverse, in part, the hyperreninemia, the hyperaldosteronism and the hypokalemia was further explored, as was the effect of such inhibition on the reabsorption of chloride in the thick ascending limb of the loop of Henle ($C_{H_2O}/C_{H_2O} + C_{Cl}$), a property which has been found to be approximately 50 percent of normal in all patients with the syndrome studied thus far. Even large doses of synthetase inhibitors failed to restore plasma potassium to normal, although they produced a higher plasma potassium than that found in the untreated state, presumably because of their ability to limit aldosterone production via the limitation of plasma renin activity. It was found that inhibition of PG synthetase has no effect on the inability to reabsorb normal amounts of chloride, and thus to produce normal amounts of free water in the loop of Henle. Accordingly, it appears that this defect is the most "proximal" one thus far uncovered in this syndrome. Presumably it leads in turn to potassium depletion, which may well produce the excess of renal prostaglandin E with the consequent hyperreninemia and the hyperaldosteronism.

Studies on the aminergic neurotransmitters, dopamine, norepinephrine, epinephrine and serotonin concern especially the study of the rate-limiting enzymes for production of these amines. The efforts to isolate tyrosine hydroxylase as an homogeneous protein continue. It appears likely that tyrosine hydroxylase, like dopamine beta hydroxylase, is a tetrameric molecule, which probably separates with the particulate fraction and is heat-labile. The activity of tyrosine hydroxylase is limited by the availability of the co-factor, tetrahydro-biopterin, and it appears that the affinity of tyrosine hydroxylase for its co-factor is controlled by cyclic AMP-dependent protein phosphorylation. *In vivo*, experiments were done supporting the view that regulation of tyrosine hydroxylase, mediated by neurotransmitter receptor activation or blockade, may depend, in turn, upon cyclic AMP dependent protein phosphorylation.

Characterization of dopamine beta hydroxylase was continued with the demonstration that both the dimer and the tetramer isolated from human plasma have catalytic activity, although the monomer does not. Catalytic activity was studied with electron paramagnetic resonance and the results suggest that the activity involves the interaction of substrate nitrogen with the enzymic copper which forms part of the DBH molecule.

Studies on tryptophane hydroxylase have continued, although it has thus far resisted total isolation. It appears that protein phosphorylation is involved in the kinetic activation of tryptophane hydroxylase.

The activation of pineal serotonin N-acetyl transferase was further studied. The steps in this activation appear to be the increase of intracellular cyclic AMP concentration induced by activation of beta receptors, the phos-phorylation of a protein within the pineal chromatin and an increase of messenger RNA for serotonin N-acetyl transferase, followed by increased production of serotonin N-acetyl transferase. The control of tyrosine hydroxylase in the rat carotid body was studied as a model for the induction of dopamine production. It was found that anoxia increases dopamine release in the carotid body, that this is accompanied by an increase of phosphodiesterase and an increase in RNA polymerase-dependent RNA synthesis. The induction of the tyrosine hydroxylase, the increase in RNA synthesis and the activation of phospho-diesterase all depend upon an intact innervation of the carotid body. It is thus likely that a sequence of events similar to that for pineal production of serotonin N-acetyl transferase occurs in the carotid body for the production of dopamine.

The role of neuronal activation and of neurotransmitters in the regulation of blood pressure was further studied in the spontaneously hypertensive rat (SHR). With age-dependent increase of blood pressure, there were found increases in tyrosine hydroxylase and dopamine beta-hydroxylase in the hypothalamus. It is thus likely that the development of hypertension is related to the amine activity in the hypothalamus. The role of sympathetic nerve activity in the development of sustained hypertension in SHR was further studied by an analysis of non-collagen protein synthesis in arteries, which is greatly accelerated during the development of hypertension. When blood pressure elevation is prevented by chlonidine, which acts centrally to prevent sympathetic outflow, the increase in non-collagen protein synthesis was also prevented. A similar effect was found with the ganglionic blockade by hexamethonium. On the other hand, when a direct vasodilator such as hydralazine was used to control blood pressure, it did not lower the non-collagen protein synthesis in the arteries as compared to that found in normal Wistar-Kyoto rats. It was further found that phenoxybenzamine, producing alpha-receptor blockade, could prevent both the blood pressure rise and the increased rate of production of non-collagen protein, whereas propranolol, producing beta-receptor blockade, did not affect either variable.

The studies of plasma norepinephrine and dopamine beta-hydroxylase as possible indices of peripheral sympathetic nerve activity in man were

continued. No clearer correlation of either of these indices with the development of idiopathic hypertension could be found. The studies were extended to include measurement of both substances in cerebrospinal fluid: these studies will be extended to include patients with various types of hypertension. A method for the determination of the hydroxylase co-factor, tetrahydrobiopterin in cerebral spinal fluid has been developed and is being extended to patients with abnormalities of blood pressure and with mental illness.

The urinary kallikrein and kinins and the plasma prekallikrein and bradykinin were studied in extenso. Low-sodium or high-potassium diets, which increase aldosterone secretion, increased urinary kallikrein, but not urinary kinin, which appeared to bear no relationship to urinary kallikrein in any study. Since the plasma bradykinin is elevated in Bartter's Syndrome, and falls to normal with indomethacin treatment, the effect of indomethacin was studied in normal subjects. It was found that it does not affect plasma bradykinin. Accordingly, the elevated plasma bradykinin in the Syndrome must be related to some other abnormality (e.g., elevated plasma renin) that is brought to normal with indomethacin. Elevation (or decreased degradation) of plasma bradykinin with angiotensin, by competition for plasma converting enzyme, could not be shown in experimental animals, nor could blockade of converting enzyme in man be shown to lower degradation of plasma bradykinin.

Whereas the activities of the Branch relative to metabolic bone disease and parathyroid function have been perforced limited, we have continued a study of the role of parathyroid hormone in the production of chronic "idiopathic" osteoporosis. All patients with idiopathic osteoporosis previously identified in the Branch are being restudied for autonomy of the parathyroid as measured by responsiveness of the cyclic AMP clearance to changes of plasma calcium. In addition, the effect of acidosis on hydroxylation of 25-hydroxycholecalciferol is being measured in an attempt to explain the rarefaction of bone which occurs in patients with acidosis.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF [illegible] PROJECT

PROJECT NUMBER
Z01-HL-01801-03 HE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Outpatient Hypertension Diagnostic Screening Program

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

PI:	A.A. Taylor	Senior Investigator	HE NHLBI
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	J.R. Gill, Jr.	Senior Investigator	HE NHLBI
	F.C. Bartter	Chief, Hyper.-Endo. Branch	HE NHLBI
	C.S. Delea	Administrative Assistant	HE NHLBI
	J.W. Cox	Biochemist (Supervisor)	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Steroid and Mineral Metabolism

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.4	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)

Random renin-sodium profiling produces heterogeneous subgroups of low, high, and normal renin essential hypertensive patients. Ten of 34 hypertensive patients (29.4%) changed renin categories on repeat examination. Renin values in hypertensive patients are distributed along a continuum from low to high rather than being divided into discrete renin subgroups.

PHS-6040

Project Description:

The application of sophisticated methods for measuring renin and aldosterone to the study of hypertensive patients has contributed to our ability to diagnose secondary forms of hypertension such as primary aldosteronism and renal artery stenosis. Several procedures for measuring renin and aldosterone in hypertensive patients under defined physiologic and/or pharmacologic conditions have been proposed.

Objective: The purpose of this study was to evaluate the accuracy of these various methods for categorizing hypertensive patients into selected subgroups based on their renin and aldosterone response to a defined stimulus. Currently the most commonly used method for such categorization relates plasma renin activity after 3 hours of upright posture to the individual's 24 hour urinary sodium and aldosterone on a random diet, the "renin-sodium-aldosterone index". Other investigators have classified hypertensive patients according to their renin response to upright posture and variable oral dosages of furosemide. Still others have examined the renin and aldosterone response to upright posture in patients who have achieved sodium balance on a low sodium diet.

Project Protocol: Each patient referred to the Hypertension Outpatient Clinic was seen by a physician who took a history and performed a physical examination. A chest x-ray, electrocardiogram, urinalysis, urine culture and routine serum, chemistries were obtained during the patient's first visit. An intravenous pyelogram and radioactive renogram were obtained between the first and second visits. Each patient was taught to take his own blood pressure and was requested to take it 6 times a day. On the morning of the second visit, the patient brought a 24 hour urine sample for sodium, potassium, creatinine, 17OHCS, 17KS and aldosterone excretion rate, and 45 min.-supine and 3 hour-upright blood samples were obtained for plasma renin activity, aldosterone and cortisol. Based on these data the patients was then placed in a diagnostic category. Normal volunteers giving informed consent were studied in a similar fashion.

Major Findings: We have evaluated 243 hypertensive patients by this method and compared their values with the renin-sodium-aldosterone index in 89 normotensive subjects. LREH with normal plasma and urinary aldosterone was found in 40% of these patients. Of the 34 hypertensive patients initially classified as having normal or low renin, 10 (29.4%) changed category when the renin-sodium index test was repeated. Several factors such as the age, sex and race of the volunteer and the amount of sodium excreted in the urine influence the "normal range" of values. Renin values in hypertensive patients are distributed along a continuum rather than being divided into discrete renin subgroups.

Significance: The renin-sodium index as a test for defining distinct low, normal or high renin subgroups of the hypertensive population is of limited usefulness.

Publication:

1. Mitchell, J.R., A.A. Taylor, C.R. Lake, D.E. Rollins and F.C. Bartter: NIH Combined Clinical Staff Conference: Renin-aldosterone profiling in hypertension. Ann. Intern. Med. 1977 (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-01803-03 HE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Aminoglutethimide in Low-Renin Essential Hypertension

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

PI:	J.R. Mitchell	Chief, Sec. on Clin. Pharm.	CP NHLBI
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COOPERATING UNITS (if any)
None

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Section on Clinical Pharmacology and Metabolism

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	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)

Blood pressure and adrenal steroid biosynthesis in 7 patients with LREH was evaluated before and after treatment with aminoglutethimide (AG) (1 gm/day for 3 weeks). AG reduced BP in all patients, decreased the excretion of aldosterone 75%, and raised plasma renin activity into the normal range, but failed to decrease plasma concentrations of progesterone, 17-hydroxy-progesterone, deoxycorticosterone, 18-hydroxydeoxycorticosterone, 11-desoxycortisol, or cortisol, or the urinary excretion of 16-beta-OH-dehydroepiandrosterone, 17-hydroxy or 17-ketosteroids. Secretion rates for 16-beta-OH DHEA were not changed by AG. The observation that chronic administration of aminoglutethimide lowered blood pressure in these patients and elevated their plasma renin activity to the normal range without decreasing production of the adrenal steroids, deoxycortico-sterone, 18-hydroxydeoxycorticosterone and 16-beta-OH dehydroepiandrosterone, makes it unlikely that the steroids are responsible either for the suppressed renin or the elevated blood pressure in patients with low renin essential hypertension.

Project Description:

Objectives: Patients were tested for the blood pressure-lowering effect of aminoglutethimide, an inhibitor of adrenal steroid synthesis. The pathways of adrenal steroid synthesis that are inhibited by aminoglutethimide in blood-pressure responsive patients, and therefore the pathways that might be mediating the hypertension, were determined.

Treatment with aminoglutethimide lowers the blood pressure of patients with hypertension secondary to primary aldosteronism or Cushing's syndrome (Gaunt, et al., Clin. Pharmacol. Therap. 9: 657, 1968; Temple and Liddle, Ann. Rev. Pharmacol. 10: 199, 1970; Fishman, et al., J. Clin. Endo. Metab. 27: 481, 1967; and Gorden, et al., J. Clin. Endo. Metab. 28: 921, 1968). In 1969 Liddle's group (Woods, et al., Arch. Int. Med. 123: 366, 1969) reported that 6 of 9 hypertensive patients with the syndrome of low-renin (normal aldosterone) essential hypertension experienced a lowering of blood pressure after administration of aminoglutethimide but patients with normal renin hypertension failed to respond. They noted also that the hypertension of low-renin patients responded to therapy with spironolactone (Carey, et al., Arch. Int. Med. 130: 849, 1972), a renal antagonist of sodium-retaining steroids, and to bilateral adrenalectomy (Gunnels, et al., Ann. Int. Med. 73: 901, 1970). This report triggered a widespread search for the excessive adrenal secretion of a sodium-retaining steroid other than aldosterone, and several groups subsequently have postulated that various steroids are of etiologic significance in low-renin hypertension (Brown, et al., Lancet ii: 243, 1972; Melby, et al., Circ. Res. 23: 11-143, 1971; Kuchel et al., Circ. Res. 23: 11-150, 1971; Messerli, et al., Proc. 56th Meeting of Endocrine Society, p. A-63, 1974; Slaton, et al., Clin. Res. 23: 45A, 1975: and Hisahsatu, et al., J. Clin. Endo. Metab. 40: 156, 1975). However, in these studies only the particular steroid of interest was examined. No attempt was made to determine secretion rates of most other adrenal steroids nor to demonstrate cause-effect relationships between increased steroid secretion and elevation of blood pressure. Indeed, the direct involvement of the proposed steroids in the pathogenesis of low-renin hypertension can be questioned, since none of the steroids has sufficient intrinsic sodium-retaining activity to be physiologically effective in the amounts apparently secreted by patients with low-renin hypertension.

The antihypertensive effects of aminoglutethimide result from its inhibition of adrenal steroid secretion and the accompanying renal loss of sodium and water; the drug has no direct effect on the kidney, the adrenergic nervous system or vascular smooth muscle in adrenalectomized animals and people (Gaunt, et al., Clin. Pharmacol. Therap. 9: 657, 1968; Temple and Liddle, Ann. Rev. Pharmacol. 10: 199, 1970; Fishman, et al., J. Clin. Endocr. Metab. 27: 481, 1967, Gorden, et al., J. Clin. Endocr. Metab. 28: 921, 1968;

Woods, Liddle, et al., Arch Int. Med. 123: 366, 1969). Thus, the secretion of a sodium-retaining adrenal steroid, be it aldosterone or an unidentified steroid, should play an important pathogenetic role in the hypertension of low-renin patient whose blood pressure responds to aminoglutethimide therapy.

Careful review of numerous biochemical studies in dogs and other animals, with sufficient clinical studies to confirm that man responds similarly, reveals that the secretion of cortisol by the adrenal zona fasciculata is only minimally affected by aminoglutethimide in endocrinologically normal subjects because of a compensatory increase in ACTH secretion (Gaunt, et al., Clin. Pharmacol. Therap. 9: 657, 1968; Temple and Liddle, Ann. Rev. Pharmacol. 10: 199, 1970; Fishman, et al., J. Clin. Endocr. Metab. 27: 481, 1967; Gorden, et al., J. Clin. Endocr. Metab. 28: 921, 1968; and Woods, Liddle et al., Arch. Int. Med. 123: 366, 1969). In contrast to the cortisol pathway, secretion of aldosterone by the adrenal zona glomerulosa remains inhibited by aminoglutethimide even after months of continuous therapy and compensatory increases in renin do not overcome the inhibition (Gaunt, et al., Clin. Pharmacol. Therap. 9: 657, 1968; Temple and Liddle, Ann. Rev. Pharmacol. 10: 199, 1970; Fishman, et al., J. Clin. Endocr. Metab. 27: 481, 1967; Gorden, et al., J. Clin. Endocr. Metab. 28: 921, 1968; and Woods, Liddle et al., Arch. Int. Med. 123: 366, 1969).

This phenomenon of preferential inhibition of aldosterone synthesis provides a powerful tool for defining the physiologic role of the steroids postulated as having etiologic significance in the genesis of low-renin hypertension. If the secretion of a particular steroid is not inhibited by aminoglutethimide therapy when blood pressure is reduced, then that steroid cannot be solely responsible for the hypertension. By the same rationale, one can determine which adrenal steroid synthetic pathways are inhibited by amino-glutethimide in blood pressure-responsive patients and therefore which pathways might be mediating the hypertension.

Methods Employed: Patients with low-renin hypertension on a normal diet were hospitalized for 3 days and secretory rates were determined by double isotope dilation assay following intravenous administration of radioactive tracer doses of ^{3}H-16-beta-hydroxydehydroepiandrosterone (1 µg, 4 µCi). Five plasma samples (10 ml each) were obtained, and urine was collected for 72 hours. Plasma DOC, 18-hydroxydeoxycorticosterone, desoxycortisol, cortisol, aldosterone, progesterone and 17-alpha-hydroxy-progesterone were determined by radioimmunoassay and urinary excretion of metabolites of cortisol and aldosterone were assayed chemically or by radioimmunoassay.

Following these control determinations, seven patients were treated with aminoglutethimide (1 g per day in divided doses) and steroid determinations were repeated after 4 days of treatment. Patients were then discharged and followed weekly in the clinic for

another 14 days. As outpatients, they recorded their blood pressure several times daily under the normal conditions of their environment. At each weekly clinic visit, blood (20 ml) was obtained for electrolytes, CBC, SGOT, glucose, urea nitrogen, aldosterone, cortisol and renin. Two patients were hospitalized after 3 weeks of therapy and the steroid secretory and excretory determinations repeated exactly as above (total duration of treatment with aminoglutethimide = 3 weeks).

Major Findings: Aminoglutethimide (AG) was administered to seven patients with low renin essential hypertension, and the antihypertensive action of the drug was compared with its effects on adrenal steroid production. In all patients aldosterone concentrations in plasma and urine were within normal limits prior to study. Mean arterial pressure was reduced from a pretreatment value of 117 $\pm$ 2 (mean $\pm$ SE) mmHg to 108 $\pm$ 3 mmHg after 4 days of aminoglutethimide therapy and further to 99 $\pm$ 3 mmHg when drug administration was stopped (usually 21 days). Body weight was also reduced from 81.6 $\pm$ 7.2 kg in the control period to 80.6 $\pm$ 7.0 kg after 4 days of drug treatment and to 80.1 $\pm$ 6.7 kg at the termination of therapy. Plasma renin activity was not significantly increased after 4 days of treatment but had risen to the normal range by the termination of aminoglutethimide therapy. Mean plasma concentrations of deoxycorticosterone and cortisol were unchanged during aminoglutethimide treatment whereas those of 18-hydroxydeoxy-corticosterone, progesterone, 17 α-hydroxyprogesterone and 11-deoxy-cortisol were increased as compared to pretreatment values. In contrast, aminoglutethimide treatment reduced mean plasma aldosterone concentrations to about 30% of control values. Excretion rates of 16β-hydroxydehydroepiandrosterone, 16-oxo-androstenediol, 17-hydroxycorticosteroids, and 17-ketosteroids and the secretion rate of 16β-hydroxydehydroepiandrosterone were not significantly altered by aminoglutethimide treatment whereas the excretion rate of aldosterone was reduced from 3.62 $\pm$ 0.5 (mean $\pm$ SE) in the control period to 0.9 $\pm$ 0.2 μg/24 hrs. after 4 days and to 1.1 $\pm$ 0.3 μg/24 hrs at the termination of aminoglutethimide treatment.

Significance: The blood-pressure-lowering action of AG in LREH probably resulted from inhibition of aldosterone synthesis. 16-beta-OH-DHEA appears unimportant in maintaining the hypertension of LREH because its secretion is not reduced when blood pressure is lowered by AG. Sodium-retaining steroids other than aldosterone are not important in LREH because no other major steroid pathway was inhibited by AG.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-01807-03 HE

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Studies in 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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Other:	A.A. Taylor	Senior Investigator	HE NHLBI
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	R.W. Bowden	Staff Associate	HE NHLBI
	H.R. Keiser	Deputy Chief	HE NHLBI
	F.C. Bartter	Chief, Hypertension-Endo. Br.	HE NHLBI

COOPERATING UNITS (if any)

J.C. Frolich and J.A. Oates, Department of Pharmacology
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LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Steroid and Mineral Metabolism

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	1.0	2.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Present evidence indicates that prostaglandins (PG) mediate hyperreninemia, hyperaldosteronism and the vascular resistance to angiotensin II (AII) in Bartter's syndrome. The findings that prolonged treatment of a patient with Bartter's syndrome with an inhibitor of PG synthetase corrected the hyperreninemia and hyperaldosteronism but failed to correct the hypokalemia suggest that hypokalemia is not the result of aldosteronism per se and that hypokalemia may be the cause of the hyperreninemia. This formulation is supported by the observation that patients with hypokalemia secondary to vomiting overproduced PGE_2, have hyperreninemia, and are resistant to the pressor effects of AII. Studies of renal function in patients with hypokalemia indicate that those with Bartter's syndrome have an impaired distal tubular reabsorption of chloride, a finding best explained as a defect in chloride reabsorption in the loop of Henle, as judged by the clearances of solute free water and solute. This defect, which is PG-independent, could be the cause of potassium loss and, therefore, a proximal cause of the Syndrome.

600

Project Description:

The role of PG in Bartter's syndrome was approached by an assessment of the effects of long-term treatment with a PG synthetase inhibitor (Ibuprofen, 3600 mg/day for four months). The results for a patient are shown in the table below:

	Serum K^+ mEq/L	Supine plasma renin activity ng/ml/hr	Aldosterone excretion ug/day	Urinary sodium mEq/day
Ibuprofen	2.4±0.1	2.7±0.2	4.3±0.3	80±5
No Rx	2.4±0.1	6.6±1.2	7.9±0.5	85±9

Thus, despite inhibition of prostaglandin synthesis a reduction in plasma renin activity (PRA) and maintenance of a normal aldosterone excretion (AER), serum K^+ was low and not different from the value observed after discontinuation of ibuprofen. These findings suggest that hypokalemia develops in Bartter's syndrome as a result of an abnormality other than an overproduction of PG, renin and aldosterone.

To assess the role of hypokalemia in the overproduction of renal PGE_2 and in the vascular resistance to AII in Bartter's syndrome, another series of patients with hypokalemia were studied. These patients who had developed their hypokalemia as a result of vomiting also exhibited hyperreninemia and aldosteronism.

	Serum K^+ mEq/L	Supine PRA ng/ml/hr	AER ug/day
Bartter's syndrome	2.2±0.2	14.5±7.5	16.5±6
Patients with Vomiting	2.7±0.4	30.5±14	12.5±7.5

In addition, the patients with hypokalemia secondary to vomiting also overproduce PGE_2 with a value in one patient of 417 ng/day (normal range 80 - 280 mg/day). These patients are also as resistant to the pressor effects of AII as are patients with Bartter's syndrome.

		Dose of AII needed to increase diastolic BP 20 mmHg ng/kg/min
Bartter's syndrome	Control	26±5.4
	Indomethacin	9.6±2.3
Patients with Vomiting	Control	102±34.2
	Treatment	12±2.5

Correction of the hypokalemia in the patients with vomiting restores the pressor response to A II to normal. Treatment of patients with Bartter's Syndrome with large doses of potassium does not correct hypokalemia, overproduction of PGE_2 or the pressor response to A II. Treatment with a PG synthetase inhibitor, indomethacin which corrects the overproduction of PGE_2, restores the pressor response to A II to normal. These findings suggest that hypokalemia decreases the pressor response to A II by virtue of its stimulation of PGE_2 synthesis.

To explore further the cause of renal potassium loss in Bartter's Syndrome, a solution of five percent dextrose and water was infused to produce a maximal water diuresis in patients with Bartter's Syndrome, in patients with hypokalemia secondary to vomiting, and in patients with lupus nephritis with normal serum potassium. Analysis of distal tubular function was performed in all three groups of patients, with the latter two groups serving as controls.

	C_{IN} ml/min	U_{osm} mOsmols/kg H_2O	$U_{NaV} + U_kV$ uEq/min	$\frac{C_{H_2O}}{C_{IN}}$ ml/min/100 ml GFR	$\frac{C_{Cl}}{C_{IN}}$	$\frac{C_{H_2O}}{C_{H_2O} + C_{Cl}}$
Bartter's Syndrome	69 ± 12	149 ± 15	734 ± 46	7.4 ± 1.6	9.2 ± 0.3	.49 ±0.8
Patients with Vomiting	78 ± 11	75 ± 8	284 ± 26	13.6 ± 2.1	1.1 ±0.4	.93 ±.04
Lupus Nephriti	82 ± 14	76 ± 7	266 ± 28	14.7 ± 2.1	2.5 ± .6	.86 ±.04

Patients with Bartter's Syndrome had higher urinary osmolalites (U_{osm}) with a mean almost twice that of the two control groups and this was explained in part, if not wholly, by a higher rate of solute excretion (sodium-plus-potassium). Solute free water excretion (C_{H_2O}/C_{IN}) was approximately half that observed in each of the control groups. As C_{H_2O} is largely formed by active reabsorption of chloride in the ascending H_2O limb of Henle's loop with sodium and potassium following passively, a major defect in C_{H_2O} as indicated by a high clearance of chloride (C_{Cl}/C_{IN}), suggests a H_2O defect in chloride reabsorption. Estimation of fractional chloride reabsorption in the distal nephrone gave a mean value of 0.49 ± 0.08 for the patients with Bartter's Syndrome compared to mean values of 0.93 ± 0.04 and 0.86 ± 0.04 for the two control groups. As this measurement of fractional reabsorption in the distal tubule, which is presumably normal, as well as in the loop of Henle, it is probably an underestimation of the degree of impairment in chloride reabsorption in the loop of Henle. Further, the

defect in chloride reabsorption is not affected by treatment with indomethacin (Fractional reabsorption before treatment 0.49 ± 0.08 versus 0.46 ± 0.06 during treatment $P < 0.1$) and is, therefore, PG independent.

The above series of observations suggest the following pathogenetic sequence of events in Bartter's Syndrome:

1) Impaired chloride reabsorption in loop of Henle.
2) Increased potassium secretion and excretion.
3) Hypokalemia
4) Increased synthesis of PGE_2, blood vessels and kidney.
5) Increased release of renin and formation of A II.
6) Decreased pressor response to A II.
7) Increased secretion of aldosterone.

Significance:

These studies provide information of the important role of prostaglandins in the control of renin release and in determination of blood pressure responsiveness to endogenous pressor substances. The results contribute to an understanding of vascular biology which in turn is fundamental to an understanding of hypertension. The ability to pinpoint a renal defect which is characteristic of Bartter's Syndrome facilitates its distinction from other hypokalemic disorders and permits a continuing search for the molecular basis which causes it.

Publications:

1. Gill, J.R., Jr., Frolich, J.C., Bowden, R.E., Taylor, A.A., Keiser, H.R., Seyberth, H.W., Oates, J.A. and Bartter, F.C. Bartter's Syndrome: A disorder characterized by high urinary prostaglandins and a dependence of hyperreninemia on prostaglandin synthesis. Amer. J. Med. 61: 43-51, 1976.

2. Bartter, F.C., Gill, J.R., Jr., Frolich, J.C., Bowden, R.E., Hollifield, J.W., Radfar, N., Keiser, H.R., Oates, J.A., Seyberth, H. and Taylor, A.A. Prostaglandins are overproduced by the kidneys and mediate hyperreninemia in Bartter's Syndrome. Trans. Assoc. Amer. Phys. 89: 77-91, 1976.

3. Bartter, F.C. Bartter's Syndrome. Urologic Clinics of North America 4: 253-261, 1977.

4. Bartter, F.C., Gill, J.R., Jr. and Frolich, C. Le Syndrome de Bartter. Neckar Symposium: Flammarion Press, Paris, 1977.

5. Bowden, R.E., Gill, J.R., Jr., Radfar, N., Taylor, A.A. and Keiser, H.R. Effect of different prostaglandin synthetase inhibitors on immunoreactive prostaglandin E excretion in Bartter's Syndrome. JAMA (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-01808-02 HE

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Suppression of Renin Secretion by Propranolol in Salt-Depleted Dogs

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

PI:	J.C.H. Yun	Staff Fellow	HE NHLBI
Other:	F.C. Bartter	Chief, Hypertension-Endocrine Branch	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Steroid and Mineral Metabolism

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

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)

d, l-propranolol was infused into salt-depleted conscious dogs at two dosages: 1 mg/kg followed by 0.60 to 0.67 mg/kg/hr, and 5 mg/kg followed by 1.57 - 1.76 mg/kg/hr. At both dosages propranolol decreased plasma renin activity (PRA), plasma aldosterone concentration, and heart rate. PRA was suppressed with the higher dosage but not with the lower dosage to values found with dietary salt loading. Mean arterial blood pressure (MABP) remained unchanged with the low-dose infusions, but decreased significantly with the high-dose infusions. The data suggest that the mechanism(s) for the increase in PRA with low-salt diets is sensitive to propranolol and that the effect of propranolol on MABP is dependent on the salt-intake and on the dose administered.

604

PHS-6040
(Rev. 10-76)

Project Description:

It is well established that salt depletion raises renin secretion in man as well as in experimental animals. The mechanism(s) for the increase in renin secretion, however, is still unknown. Recently we demonstrated that infusion of propranolol causes suppression of renin secretion in anesthetized as well as conscious dogs. The present investigation was to examine whether infusion of propranolol can suppress the elevated renin secretion induced by low-salt diets to the extent that they are lowered by salt loading.

Methods Employed:

Trained female mongrel dogs were placed in metabolic cages on constant diets. Urine was collected daily and urinary sodium and potassium concentrations were determined. During the first week of study, the dogs were maintained on a diet containing 60 mEq/day of sodium. Then they were placed on operating tables for the recording of blood pressure and collection of blood samples. An hour later, arterial blood samples were collected for the determination of plasma renin activity (PRA), renin substrate concentration (RS), plasma aldosterone concentration and electrolytes.

The animal was then maintained on a diet containing 4 mEq/day of sodium for three weeks. During the first two days, each dog received an injection of Thiomerin (125 mg per day I.M.). After one week on the 4 mEq sodium diet, the dogs again were placed on the operating tables, an arterial blood sample was taken and saline (1.0 mg/kg) was then injected through a catheter in a femoral vein; this was followed by a slow infusion (0.19 ml/min) of saline. Arterial blood samples were obtained every 30 minutes for 90 minutes during the infusion and also 60 minutes after the infusion had been stopped.

On the second week of the 4 mEq sodium diet, d,l-propranolol (1 mg/kg, followed by 0.60 - 0.67 mg/kg/hr) was infused into the dog instead of saline. On the third week of 4 mEq sodium diet, the same procedures were repeated except that the dose of propranolol was increased to 5 mg/kg followed by 1.57 - 1.76 mg/kg/hr.

The animals were then maintained on a diet containing 180 mEq/day of sodium for 4 weeks. On the last day of the 180 mEq sodium diet, the procedures done on the 60 mEq sodium diet were repeated. PRA was measured by radioimmunoassay for angiotensin I.

Major Findings:

Infusion of propranolol (1 mg/kg followed by 0.60 - 0.67 mg/kg/hr) caused mean PRA to decrease from 32.2 $\pm$ 6.5 ng/ml/hr (M $\pm$ SEM) during control periods to 10.7 $\pm$ 3.0 ng/ml/hr after 90 minutes of infusion ($P < 0.025$). This PRA was still significantly higher than the value of 3.2 $\pm$ 0.8 ng/ml/hr found in the same dogs on the high-salt diet ($P < 0.05$). Infusion of propranolol at the higher dosage (5 mg/kg followed by 1.57 - 1.76 mg/kg/hr) caused PRA to decrease from 26.0 $\pm$ 4.4 ng/ml/hr during control periods to 5.9 $\pm$ 1.3 ng/ml/hr after 90 minutes of infusion ($P < 0.005$). PRA continued to fall even after the

infusion had been stopped; sixty minutes later mean PRA was 2.7 ± 0.5 ng/ml/hr which was not significantly different from the value of 3.2 ± 0.8 ng/ml/hr found in the same dogs on the high-salt diet. The data suggest that the mechanism(s) for the increase in PRA with low-salt diets is sensitive to propranolol.

Publications:

1. Yun, J. C. H., Delea, C. S., Bartter, F. C. and Kelly, G.: Increase in renin release after sinoaortic denervation and cervical vagotomy. Amer. J. Physiology 230 (3): 777-783, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZL01-HL-01809-03 HE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Prostaglandins and renal function

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

PI:	J.R. Gill, Jr.	Senior Investigator	HE NHLBI
OTHER:	B.H. Brouhard	Guest Worker	HE NHLBI
	G. Kelly	Technician	HE NHLBI
	F.C. Bartter	Chief, Hypertension-Endocrine Br.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Steroid and Mineral Metabolism

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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)

In hypophysectomized dogs, renal arterial infusion of bradykinin, 5 mg/min, during a stable water diuresis increased cortical (C_{PAH}) and non-cortical plasma flow (NCPF) and sodium excretion ($U_{Na}V$). The increase in $U_{Na}V$ could be explained, in part, by an increase in delivery of proximal tubular fluid to the distal nephron $C_{H_2O}+C_{cl}/C_{IN}$ and in part, by an inhibition of distal chloride reabsorption $C_{H_2O}/C_{H_2O}+C_{cl}$. Inhibition of prostaglandin (PG) synthesis for three days significantly decreased free water formation C_{H_2O}/C_{IN} and $C_{H_2O}+C_{cl}$ C_{IN} such that net $U_{Na}V$ was not different. Infusion of bradykinin did not change C_{PAH} but did increase NCPF, $C_{H_2O}+C_{cl}/C_{IN}$, inhibit $C_{H_2O}/C_{H_2O}+C_{cl}$ and increase $U_{Na}V$ as in control studies. Thus the effects of bradykinin on C_{PAH} appear to be mediated by PG, whereas the effects of bradykinin on NCPF, tubular reabsorption of sodium appear to be PG-independent.

607

PHS-6040
(Rev. 10-76)

Project Description:

Previous studies indicate that bradykinin stimulates the synthesis of PGE by the kidney in vivo as well as by renal interstitial cells in tissue culture. As renal arterial infusion of PGE or bradykinin produces vasodilatation and natriuresis, the following studies were done to determine if bradykinin exerts its renal effects by stimulation of PGE synthesis.

Water diuresis was produced in anesthetized hypophysectomized cortisol treated dogs by infusion of 2.5 percent dextrose. When urine flow was steady, control clearance measurements were obtained and bradykinin 5 ug/min was infused into left renal artery for five 20-minute periods. Similar studies were performed in dogs treated with indomethacin 75 mg/day, for three days to inhibit synthesis of PG.

The table below presents the effect of bradykinin alone:

	MAP mmHg	C_{PAH} ml/min	NCPF ml/min	$\frac{C_{H_2O}+C_{cl}}{C_{IN}}$ ml/min/ 100ml GFR	$\frac{C_{H_2O}}{C_{H_2O}+C_{cl}}$	$U_{Na}V$ µEq/min
Pre-infusion						
Left	131±6	88±8	76±11	6.5±0.9	.94±.02	14±5
Right		91±9	-	6.1±0.5	.96±.01	11±3
Bradykinin						
Left	134±5	109±9*	132±43*	11.0±1.5*	.69±.06	144±30*
Right		78±7	-	6.0±0.5	.97±.01	9±2
Post-infusion						
Left	138±5	80±8	62±11	4.9±0.6	.95±.01	20±3
Right		81±11	-	6.1±0.5	.96±.01	14±3

*$P < 0.05$, when left kidney compared to the right kidney except in case of NCPF, the infused left kidney is compared to the pre-infusion left kidney.

Bradykinin significantly increased cortical (C_{PAH}) and non-cortical plasma flow (NCPF), increased distal delivery, inhibited distal fractional chloride reabsorption $\frac{C_{H_2O}}{C_{H_2O}+C_{cl}}$ and increased the excretion of sodium. These findings suggest that bradykinin produces vasodilatation of cortical and medullary vessels and inhibits tubular reabsorption of sodium chloride in both the proximal and distal nephron. The effects of bradykinin in dogs treated with indomethacin are presented in table below:

	MAP	C_{PAH}	NCPF	$\frac{C_{H_2O}+C_{cl}}{C_{IN}}$	$\frac{C_{H_2O}}{C_{H_2O}+C_{cl}}$	$U_{Na}V$
	mmHg	ml/min	ml/min	ml/min/ 100ml GFR		μEq/min
Pre-infusion						
Control	131±6*	88±8	76±11	6.5±0.9	.94±.02	14±5
Indomethacin	152±5	67±7	61±15	3.6±0.4*	.87±.03	10±4
Bradykinin						
Control	134±5	109±9*	132±43	11.0±1.5	.69±.06	144±30
Indomethacin	150±4	72±9	120±29	10.6±1.5	.60±.06	122±15
Post-infusion						
Control	138±5	80±8	62±11	4.9±0.6	.95±.01	20±3
Indomethacin	164±7	56±10	52±20	4.6±0.8	.84±.09	15±4

*$P < 0.05$, when left kidney of control series is compared to left kidney of indomethacin treated series.

Treatment with indomethacin resulted in a significantly higher mean arterial pressure and a lower delivery of tubular fluid from proximal to distal nephron. Also not shown is a significantly lower free water clearance $\frac{C_{H_2O}}{C_{IN}}$ 3.2 ± 0.45 versus 6.0 ± 0.9 ml/min/100ml GFR. In previous studies infusion of indomethacin was associated with a decrease in $\frac{C_{H_2O}}{C_{IN}}$ and an increase in $U_{Na}V$. If the initial loss of sodium were sustained during prolonged treatment with indomethacin, it could result in the increase in proximal tubular reabsorption of sodium and curtailment of sodium excretion observed after three days of treatment. Infusion of bradykinin produced increases in NCPF, in $\frac{C_{H_2O}+C_{cl}}{C_{IN}}$, and in $U_{Na}V$ as in the control studies, but differed in that it failed to increase C_{PAH}. Thus the findings that indomethacin blocks the effects of bradykinin on cortical blood flow indicate that this effect of bradykinin is mediated by PG. The effects of bradykinin on NCPF and sodium excretion appear to be PG independent, however.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-01812-02 HE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Inpatient Hypertensive Diagnostic Studies

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

PI:	A.A. Taylor	Senior Investigator	HE NHLBI
OTHER:	J.R. Mitchell	Chief, Sec. on Clin. Pharm.	SCPM DIR NHLBI
	C.R. Lake	Senior Investigator	LCS NIMH
	F.C. Bartter	Chief, Hyper.-Endo. Branch	HE NHLBI
	C.S. Delea	Administrative Assistant	HE NHLBI
	J.W. Cox	Biochemist (Supervisor)	HE NHLBI
	J.L. Pool	Staff Associate	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Steroid and Mineral Metabolism

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	1.5	1.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Renin-aldosterone profiling was used to classify patients with hypertension: a) 243 patients with essential hypertension were classified by renin-urinary sodium indexing, b) 107 were reclassified by response to administration of furosemide and intravenous saline, c) 45 were further classified by response to a low sodium diet. Arbitrary "normal ranges" were determined in 89, 32, and 38 volunteers, respectively. The possibility is considered that low renin patients have a primary renal abnormality in renin secretion with relative deficiencies of angiotensin II and aldosterone when they are subjected to diuresis. They can maintain aldosterone secretion under normal conditions because their adrenal aldosterone receptor is supersensitive to angiotensin II. No evidence of abnormal sympathetic neural activity was found among the renin subgroups. Renin-aldosterone profiling in clinical practice is useful mainly in the detection of patients with curable forms of secondary hypertension. Aldosterone/renin ratios obtained while a patient's extracellular fluid volume is expanded and contracted may be diagnostically helpful.

610

Project Description:

Objectives: Measurements of renin and aldosterone during various states of volume expansion and contraction have been used to detect patients with secondary forms of hypertension and to subdivide hypertensive patients into low, normal and high renin categories (Grim et al. JAMA 237: 1331, 1977). This study compared several renin profiling techniques in hypertensive patients and normal volunteers to: 1) evaluate their diagnostic accuracy in detecting primary aldosteronism and renovascular hypertension, 2) determine the reproducibility of such tests in classifying hypertensive patients into renin subgroups, 3) to compare plasma norepinephrine concentrations, as an index of sympathetic nervous system activity, with the renin response to volume expansion and contraction in hypertensive patients in low, normal and high renin subgroups.

Major Findings: Essential Hypertension: Different subgroups of hypertensive patients were defined from measurements of plasma renin activity and plasma and urinary aldosterone concentrations plotted against daily urinary sodium excretion as an approximation of the patient's state of sodium balance. The normal ranges of plasma renin activity, plasma aldosterone and 24-hr urinary aldosterone excretion were derived from 126 determinations from 89 normal volunteers.

Besides the renin-sodium indexing method, different subgroups of patients were defined from measurements of plasma renin activity and plasma and urinary aldosterone concentrations after volume contraction and volume expansion (furosemide, 40 mg and 120 mg, 10 mEq sodium diet, and intravenous saline infusion). A total of 107 patients and 32 normal volunteers underwent the furosemide and saline tests, and 45 patients and 38 normal volunteers underwent the 10 mEq sodium balance test.

Similar to reports by other investigators (Laragh et al., Am J Med 52: 633, 1972), we found hyperbolic relations between urinary sodium excretion and plasma renin activity and plasma and urinary aldosterone concentrations. For several reasons, however, the urinary sodium indexing approach was unsatisfactory as the sole procedure for renin-aldosterone profiling. First, low renin patients could not be reliably detected without major restriction of dietary sodium.

Second, the "normal range" derived from the study of 89 volunteers was much greater than the normal range obtained in studies with smaller number of volunteers (Brunner et al. NEJM 284: 441, 1972). It was particularly distressing that our "normal range" for the low renin category changed progressively with the addition of 23 volunteers between 41 and 74 years of age. A decrease in plasma renin activity and aldosterone excretion with increasing age was also apparent in the 45 patients and 38 normal volunteers studied after achieving equilibrium on a 10 mEq sodium diet. Thus, the establishment of

a "normal range" to determine renin subgroups of patients was arbitrary and highly dependent upon the age composition of the normal volunteer population.

Third, although the different approaches to identification of renin subgroups readily identified extreme aberrations, many patients were classified in different subgroups by the various tests. It was apparent that the renin subgroups, in fact, were not discrete populations but represented a continuum of values and the use of arbitrary "normal ranges" to define each subgroup led to some overlapping. This probably explains why renin subgrouping differs occasionally even when the same test is repeated in the same patient and when the same patient is evaluated by the different tests. For example, when 34 of our patients with normal or low renin hypertension by renin-sodium classification underwent a repeat test, seven remained in the low renin subgroup and 17 remained in the normal subgroup, but 10 (29.4%) changed categories from low to normal (eight patients) or from normal to low (two patients). Similarly, when 20 patients with low renin hypertension by renin-sodium classification were grouped according to their response to dietary sodium restriction 11 (55%) changed categories from low renin to normal renin hypertension. In contrast, when 26 patients with low and normal renin hypertension by response to furosemide were grouped according to their response to dietary sodium restriction, only four (15.4%) changed categories, all switching from the low to normal renin subgroup. Therefore, categorization of patients into renin subgroups is dependent on the test used, and stimulation of the renin system by volume restriction may identify a more homogeneous group of low renin patients, namely those with the most hyporesponsive renin system.

Fourth, although our experience with the high renin subgroup consisted of fewer patients than in the low and normal subgroups, our impression again was that a more homogeneous group was identified when patients were tested supine after volume expansion by saline infusion or by high dietary sodium intake. Thus, suppression of the renin system may be a better method of identifying patients with the most responsive renin system.

Fifth, the degree of variation in plasma renin activity or in plasma or urinary aldosterone concentrations plotted against 24-h urinary sodium excretion seemed considerably greater in the clinic patients and normal volunteers taking random sodium diets as compared to hospitalized patients. Thus, the renin-sodium index approach to renin subgrouping would appear most reliable when patients are taking a constant sodium intake, a state often difficult to achieve in an outpatient setting.

A major abnormality identified in patients with low renin essential hypertension was an exaggerated ratio of plasma aldosterone or urinary aldosterone to plasma renin activity. This high aldosterone

production has led to the suggestion that low renin hypertension may represent a variant of primary aldosteronism (Grim et al., J. Clin. Endocrinol. Metab. 39: 247, 1974). However, the secretion of aldosterone in our low renin patients was not autonomous because volume expansion with saline decreased aldosterone values to the normal range.

Moreover, these patients in fact develop relative aldosterone deficiency in comparison to normal volunteers or to other patients with essential hypertension when undergoing volume contraction by administration of furosemide or by dietary sodium restriction.

Plasma norepinephrine concentrations were not different among volunteers and any of the renin subgroups of hypertensive patients whether subjects were supine or standing and whether renin subgrouping of patients was by the saline and furosemide tests or by the less selective renin-sodium indexing.

Secondary Hypertension: Measurements of renin and aldosterone were usually but not always diagnostic in 11 patients with primary aldosteronism. In contrast, these patients could be consistently distinguished from those with essential hypertension because of a markedly elevated ratio of plasma or urinary aldosterone to plasma renin activity.

Abnormally high supine plasma renin values after saline infusion in a limited number of patients with renovascular hypertension suggests that this test may be useful for deciding which hypertensive patients to evaluate by renal angiography and by renal vein renin measurements.

Significance: Same as above.

Publications:

1. Mitchell, J.R., Taylor, A.A., Pool, J.L., Lake, C.R., Rollins, D.E., and Bartter, F.C.: NIH Combined Clinical Staff Conference: Renin-aldosterone Profiling in Hypertension. Ann. Intern. Med. 1977 (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-01813-02 HE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Effect of Angiotensin on Aldosterone

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

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	F.C. Bartter	Chief, Hyper.-Endo. Branch	HE NHLBI
	J.L. Pool	Staff Associate	HE NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Steroid and Mineral Metabolism

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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)

Patients with low renin essential hypertension (LREH) have an exaggerated ratio of plasma or urinary aldosterone to plasma renin activity while taking an unrestricted sodium diet or during volume contraction with furosemide or dietary sodium restriction. We have tested the hypothesis that this abnormal aldosterone/renin ratio in LREH results from an increased sensitivity of the adrenal receptor for aldosterone synthesis to angiotensin II (AII) by administering A II intravenously to 20 normal volunteers and 24 patients with low (9) or normal (15) renin hypertension. Patients with LREH have greater increases in plasma aldosterone concentrations and diastolic blood pressure per unit of A II infused than do normal volunteers or patients with normal renin essential hypertension. This adrenal supersensitivity to A II is not compatible with a primary adrenal defect causing LREH.

614

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Patients with low renin essential hypertension demonstrate an exaggerated ratio of plasma or urinary aldosterone per unit of plasma renin activity during ad libitum sodium sodium sodium intake or after volume constriction by furosemide or equilibration on a 10 mEq/day sodium intake yet they have lower plasma or urinary aldosterone concentrations in response to such maneuvers than do other patients with essential hypertension. One explanation for these observations would be an increased sensitivity to angiotensin II at the adrenal receptor for aldosterone synthesis in LREH patients. This hypothesis is tested in the present study by determining the blood pressure and aldosterone response to angiotensin II in normal volunteers and patients with low and normal renin hypertension.

Methods Employed: Volunteers and patients underwent an extensive clinic evaluation including a complete history and physical exam, chest x-ray, electrocardiogram, urinalysis, electrolytes, creatinine clearance and characterization of their renin angiotensin system. Those patients who did not have renal failure, congestive heart failure, angina pectoris, history of prior myocardial infarction, central nervous system vascular disease, cardiac arrhythmias or malignant hypertension were admitted to the 4 day study.

Day 1,2: Subjects were given a 109 Na^+, 70 K^+ diet. On day 2, subjects were kept supine and 12 ml blood was obtained at 0, 60 and 120 minutes for plasma aldosterone, cortisol, and renin potassium.

Day 3: Angiotensin II was infused at a fixed dose (2 ng/kg/min) for 1 hour with the patient supine and monitored by blood pressure determinations every 2 minutes and by continous electrocardiogram. Blood was withdrawn via a venous heparin lock at 0, 30, 45, 60 and 120 minutes for plasma aldosterone, cortisol, renin and potassium determinations.

Day 4: Angiotensin II was infused in a dose-pressor response manner until diastolic blood pressure was raised by 20 mmHg. This dose was then infused for 1 hour under the above conditions and blood samples (12 ml) were again obtained at 0, 30, 45, 60 and 120 minutes. No patient's blood pressure was allowed to exceed 190 mmHg systolic or 120 diastolic.

When angiotensin was infused at a fixed dose of 2 ng/kg ideal body weight/min for 60 min, the increase in plasma aldosterone concentrations above basal values was greatest in the low renin patients. This exaggerated aldosterone response was also apparent when a dose of angiotensin II sufficient to raise the diastolic blood pressure by 20 mmHg was infused for 60 min. Neither plasma cortisol nor potassium concentrations were increased by angiotensin II at any of the doses used in these studies.

These data are consistent with the hypothesis that patients with the syndrome of low renin essential hypertension have a supersensitivity to angiotensin II at the adrenal receptor for aldosterone synthesis. This hypothesis is supported by the report that plasma angiotensin II concentrations are proportional to plasma renin activity in low renin and other patients with hypertension, demonstrating directly that low renin patients have neither an increased generation nor a decreased catabolism of angiotensin II (Beevers, Brit. Med. J. 1: 415, 1977).

Significance: Same as above

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-0814-02 HE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
The effect of triamterene on blood pressure, the renin-aldosterone axis and sex steroids.

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

PI:	A.A. Taylor	Senior Investigator	HE NHLBI
OTHER:	J.R. Mitchell	Chief, Section of Clin. Pharm.	SCPM DIR NHLBI
	D.E. Rollins	Research Associate	SCPM DIR NHLBI
	R.J. McMurtry	Clinical Associate	SCPM DIR NHLBI
	J.L. Pool	Staff Associate	HE NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine Branch

SE
Steroid and Mineral Metabolism

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.2	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)

The ability of triamterene to control blood pressure, alter the renin-aldosterone axis and cause clinical and biochemical side effects was assessed. In addition, because of the many undesirable side effects of spironolactone, triamterene was evaluated as a suitable alternative potassium-sparing diuretic in the treatment of hypertension. Triamterene was not an effective antihypertensive agent despite presumed volume depletion in patients as reflected by an increase in plasma renin activity and aldosterone excretion rate. There was also a significant but reversible deterioration in renal function in 20 patients treated with triamterene as the dose was increased.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The purpose of this study was to assess the ability of triamterene to control blood pressure, to alter the renin-aldosterone axis, and to cause clinical and biochemical side effects. In addition, because the only other major potassium-sparing diuretic, spironolactone, has many undesirable side effects, triamterene was evaluated as a possible alternative to spironolactone in the treatment of hypertension.

Methods Employed: Blood pressure was measured while patients were hospitalized and when seen in the outpatient clinic. Patients also took their own blood pressure at home and considerable weight was given to their home blood pressure records in evaluating their treatment. Routine blood and urine tests were done by the NIH Clinical Laboratories and hormone assays (renin, aldosterone, cortisol and sex steroids) were performed by Hazleton Laboratories.

Major Findings: Triamterene failed to lower blood pressure to the normotensive range in most hypertensive patients. Several patients noted gastrointestinal upset initially which tended to abate with time, and almost every patient noted the development of significant thirst. Renal function deteriorated in 20 patients treated with the drug, but returned to pretreatment status when triamterene was discontinued. Serum potassium rose from pretreatment hypokalemia (in those who have previously taken diuretics) to normokalemia while normokalemia was maintained in the remainder with one exception. This patient, who had a significant fall in creatinine clearance, developed mild hyperkalemia. There was a significant and gradual rise in plasma renin activity in all patients with continued therapy. There was no change in sex steroid levels. Two patients developed a drug related rash.

Significance: Triamterene alone is not an effective antihypertensive agent in most patients with hypertension despite increases in PRA and AER presumed to be due to volume depletion. In addition, its use in increased doses is associated with significant but reversible deterioration in renal function.

Publications:

1.. Mitchell, J.R., A.A. Taylor, C.R. Lake, D.E. Rollins and F.C. Bartter: NIH Combined Clinical Staff Conference: Renin-aldosterone profiling in hypertension. Ann. Intern. Med. 1977 (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-01815-02 HE

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Role of Prostaglandins in the Control of Renin Secretion in the Dog (II).

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

PI:	J.C.H. Yun	Staff Fellow	HE NHLBI
Other:	F.C. Bartter	Chief, Hypertension-Endocrine Branch	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Steroid and Mineral Metabolism

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	0.7	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 role of prostaglandins in the control of renin secretion was examined in "non-filtering kidneys" of anesthetized dogs. Infusion of indomethacin into the artery of "non-filtering kidneys" (0.5 mg/kg followed by 388 μg/min) caused a significant increase in mean arterial blood pressure (MABP) and a significant decrease in arterial plasma renin activity (PRA) when renal perfusion pressure was maintained relatively constant. Subsequent infusion of PGE_2 (1.03 μg/min) into these "non-filtering kidneys" caused a significant increase in arterial PRA without significant changes in MABP. The data suggest that PGE_2 plays a role in the control of renin secretion.

619

Project Description:

Although much work has been done on the role of prostaglandins in renal function, the role they play in the control of renin secretion is still unsettled. Vander reported that infusion of prostaglandin secretion is still renal artery of the dog caused no change in renin release. Werning et al., and Yun et al., reported that infusion of PGE_1 and PGE_2 into the renal artery of the dog causes increases in renin secretion. Since there was a natriuresis following the infusion of PGE_1 or PGE_2 in these studies, the increase in renin secretion may have resulted from the natriuresis and subsequent plasma volume contraction. If prostaglandins play a role in the control of renin secretion, infusion of an inhibitor of prostaglandin synthetase into the renal artery should cause a decrease in renin secretion and infusion of prostaglandins into the kidney should cause an increase in renin secretion. Furthermore, if the increase in renin secretion following the infusion of prostaglandins is independent of natriuresis and plasma volume contraction, infusion of prostaglandins into "non-filtering kidneys" should also cause an increase in renin secretion since there will be no or very little glomerular filtration taking place and therefore very little tubular fluid exposed to the macula densa cells, and no fluid loss. The present investigation was to examine this possibility.

Methods Employed:

Mongrel dogs were used in the experiments. "Non-filtering kidneys" were prepared according to the method of Blain et al. The dogs were anesthetized with Nembutal and ventilated with a respirator. Plasma renin activity (PRA) was determined by radioimmunoassay for angiotensin I.

Major Findings:

Infusion of indomethacin into the renal artery of "non-filtering kidneys" (0.5 mg/kg followed by 388 μg/min) caused a significant increase in mean arterial blood pressure (MABP) and a significant decrease in arterial PRA when renal perfusion pressure was maintained relatively constant. Arterial PRA was 6.21 ± 1.94 ng/ml/hr during control periods and was 3.57 ± 1.04 ng/ml/hr ($P < 0.05$) after 80 minutes of infusion of indomethacin. Subsequent infusion of PGE_2 (1.03 μg/min) into these "non-filtering kidneys" caused a significant increase in arterial PRA to 12.98 ± 3.94 ng/ml/hr ($P < 0.05$) after 60 minutes of infusion of PGE_2. The data suggest that PGE_2 plays a role in the control of renin secretion.

Publications:

1. Yun, J. C. H., Kelly, G., Bartter, F. C., Smith, H., Jr.: Role of prostaglandins in the control of renin secretion in the dog. Circ. Res. 40 (5): 459-464, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER
-		Z01-HL-01816-01 HE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Simultaneous determination of propranolol and 4-OH propranolol by HPLC

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

PI:	A. Taburet	Visiting Fellow	SCPM DIR NHLBI
Others:	A. Taylor	Senior Investigator	HE NHLBI
	J.R. Mitchell	Chief, Section on Clin. Pharmocology	SCPM DIR NHLBI
	D.E. Rollins	Staff Fellow	SCPM DIR NHLBI
	J.L. Pool	Staff Fellow	HE NHLBI

COOPERATING UNITS (if any)
HEB, NHLBI

LAB/BRANCH
Office of the Director of Intramural Research

SECTION
Section on Clinical Pharmacology and Metabolism

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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)

A quantitative method for the simultaneous measurement of propranolol and its major metabolite, 4-hydroxypropranolol, in human plasma has been developed. Following their separation by high pressure liquid chromatography as little as 1 ng of propranolol and 5 ng of 4-OH propranolol can be measured using a fluorimetric detector. Interference with the assay by other commonly used cardiovascular drugs was studied. The plasma concentrations of propranolol and 4-hydroxypropranolol were measured in hypertensive patients following oral administration of various dosages of propranolol.

621

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: After oral administration of propranolol, a metabolite 4-hydroxypropranolol is found in plasma which has adrenoreceptor blocking activity equipotent to that of propranolol. The only published method which simultaneously measured both propranolol and 4-hydroxypropranolol (Walle et al., J. Chromatogr. 114, 351-359, 1975) used gas chromatography-mass spectroscopy, a specific and sensitive but time-consuming procedure. We have developed an alternative method using HPLC separation and fluoremetric detection of propranolol and 4-OH propranolol which is more rapid than GC-MS without sacrificing specificity or sensitivity.

Method Employed: A HPLC method was developed. Since the two compounds to be separated were weak bases, a paired-ion chromatography was employed with a reverse phase column. The counter ion was 1-heptane sulfonic acid (.005 M). The best separation was achieved with a solvent mixture containing 60% methanol, 39% water, 1% glacial acetic acid and .005 M heptane sulfonic acid. The flow rate of the mobile phase was kept constant at 1 ml/min. The 4-hydroxypropranolol being more polar was eluted before propranolol.

A high sensitivity was obtained with a fluoremetric detector. The maximum excitation and emission spectra of fluorescence was found to be different for propranolol and 4-OH propranolol. Two sets of primary and secondary filters were used, one for the 4-hydroxypropranolol, the other for propranolol measurement. The addition of internal standards (pronethalol for propranolol and labetalol for 4-hydroxypropranolol) allowed accurate quantitative analysis. Two ml of plasma was extracted at pH 7.5 with ether. The ether phase was evaporated to dryness and the sample was dissolved in 200 μl of the solvent mixture. Of the 200 μl, 100 μl was injected to measure 4-hydroxypropranolol concentrations and after changing the filters, 40 to 60 μl was injected to determine propranolol concentrations.

The interferences of other commonly used cardiovascular drugs were also studied. Only triamterene and quinidine were found to interfere, due to their high fluorescence.

Three patients were given oral doses of propranolol four times daily for three days. The first dose was 20 mg, the others 80 mg. Blood samples were collected for periods of 6 hours; the first day after the 20 mg and the first 80 mg dose, the second and third days after the 80 mg morning dose.

Major Findings: The calculation of the areas under the curve for each 6 hours of sampling show that a steady state of propranolol was reached at the end of the experiment. The 4-OH levels remained low. No significant change could be seen either when increasing propranolol dose or when the steady state is reached. No relationship was found between 4-OH propranolol and propranolol levels.

Significance: A rapid, specific and sensitive HPLC method for simultaneously measuring propranolol and 4-OH propranolol will allow further studies of contributions of each compound to beta-adrenergic blocking activities in man.

Publications: none.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-01817-01 HE

PERIOD COVERED

July 1, 1976 to Sept. 30, 1977

TITLE OF PROJECT (80 characters or less)

The Quantitative Determination of Acetaminophen Conjugates by HPLC

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

P.I.	A.R. Buckpitt	Guest Worker	SCPM DIR NHLBI
	D.E. Rollins	Research Associate	SCPM DIR NHLBI
	J.R. Mitchell	Chief, Section on Clinical Pharm. and Metabolism	SCPM DIR NHLBI

COOPERATING UNITS (if any)

Dr. Rollins is a Research Associate in the Pharmacology-Toxicology Program, NIGMS. Dr. Buckpitt holds an NIH Research Fellowship in NHLBI.

LAB/BRANCH

Office of the Director of Intramural Research

SECTION

Clinical Pharmacology and Metabolism

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

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)

A specific, rapid and sensitive procedure for the quantitative determination of several thiol conjugates of acetaminophen from microsomal incubations has been developed. This method is capable of measuring as little as 5 ng of the glutathione, cysteine, N-acetylcysteine, cysteamine or α-mercaptopropionylglycine conjugates of acetaminophen.

624

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Previous investigations in this laboratory have indicated acetaminophen is metabolically activated by the cytochrome P-450 system to a reactive intermediate which covalently binds to tissue macromolecules. This reactive intermediate can be "trapped" in vitro with the nucleophiles GSH, cysteine or N-acetylcysteine.

Previous methods for either the quantitation or the collection of sufficient quantities of these "trapped" metabolites for mass spectral studies have been laborious and imprecise. Therefore the current HPLC method was developed in an attempt to correct these deficiences.

Methods Employed: Standard procedures for the preparation of liver microsomes and for microsomal incubations have been employed. ^{14}C-ring-acetaminophen has been used as the substrate. Microsomal incubations were quenched by the addition of ice cold methanol, the protein pellet was removed by centrifugation and an aliquot of the supernatant was taken to dryness under nitrogen. The sample was reconstituted with water and an aliquot was injected onto a C_{18} μ Bondapak reverse phase column using 12.5% Methanol/1% acetic acid/86.5% water as the mobile phase.

Major Findings: The retention times of acetaminophen, and its cysteine, glutathione, N-acetylcysteine, and α-mercaptopropionyl glycine conjugates are 9.2, 8.2, 12.1, 20.1 and 22.0 min, respectively.

Significance to Biomedical Research and the Program of the Institute: This HPLC method represents a major step in facilitating studies on the structure of the trapped reactive metabolite of acetaminophen and on the role of glutathione in the hepatic detoxification of acetaminophen.

Proposed Course of Project: Completed.

Publications: A.R. Buckpitt, D.E. Rollins, S.D. Nelson, R.B. Franklin and J.R. Mitchell: Quantitative Determination of the Glutathione, Cysteine and N-Acetylcysteine Conjugates of Acetaminophen by High Pressure Liquid Chromatography. Analytical Biochem, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-01818-01 HE

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

The relation of the hypertension of patients with sodium sensitive hypertension to cardiac output and to the adrenergic nervous system.

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

PI:	T. Fujita	Visiting Scientist	HE NHLBI
OTHER:	F.C. Bartter	Chief, Hyper.-Endoc. Branch	HE NHLBI
	C.R. Lake	Staff Psychiatrist	LCS NIMH
	W.L. Henry	Senior Investigator	CB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Steroid and Mineral Metabolism

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.8	0.8	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)

"Salt-sensitive" hypertensive patients cannot excrete sodium "loads" as readily as those with "non-salt-sensitive" hypertension. The increment of retained sodium, with a corresponding increase in blood volume, leads to an increase in cardiac output as measured by echocardiography, and is associated with a greater rise of blood pressure in salt-sensitive patients. The mechanism by which dietary sodium influences blood pressure in the two groups is not related to changes in plasma renin activity or aldosterone with salt loads, but may be due to that of the response of autonomic nervous system to these loads; in non-salt-sensitive patients autonomic "drive" may be more easily lowered by salt-loads, as compared with salt-sensitive patients in whom the persistance of such drive contributes to the rise of blood pressure.

626

PHS-6040
(Rev. 10-76)

Project Description:

In a previous study, patients with hypertension were classified as salt-sensitive or non-salt-sensitive in relation to changes in blood pressure with changes in sodium intake. On the high-Na diet, there was no statistically significant differences in plasma Na, K, Cl, CO_2, aldosterone, cortisol, plasma renin activity or urinary potassium, aldosterone or 17-hydroxycorticosteroids between the two groups. The sodium-sensitive patients retained more sodium on this diet than did the non-sodium-sensitive patients.

On the low Na diet, plasma renin activity, plasma aldosterone and urinary aldosterone were higher in the non-salt-sensitive patients. Among, the factors responsible for the increase in blood pressure may be an increase cardiac output, or differences in autonomic activity.

Levels of the neurotransmitter of the sympathetic nervous system (SNS), norepinephrine (NE), and its synthetic enzyme, dopamine-B-hydroxylase (DBH) are indistinguishable between normotensive and hypertensive subjects of the same age (Lake et al., N. Engl. J. Med., Jan. 27, 1977: Pedersen and Christensen, Acta Med. Scand., 198, 1975).

Adrenergic neuroreceptor sites, both alpha and beta, can now be evaluated on peripheral blood elements and compared to these receptors in age- and sex-matched controls. If either alpha receptors in vessels or beta receptors in heart are supersensitive to NE, it might explain the increased pulse rate and/or blood pressure in some hypertensive patients. A hypersensitivity to NE in hypertension has been demonstrated in man (Doyle and Black, 1955; Duff, 1957; Mendlowitz, 1973; and Bloom et al., 1976). By evaluating the number of receptors and their response to agonists, it may be possible to predict the response of receptors in other target organs.

In addition, patients who have large within-day circadian rhythms in their blood pressure will have cardiac outputs and catecholamines measured at the times of the peak and nadir of the circadian curve.

Methods Employed:

Patients with blood pressure at some time of day about 150/90 with no known etiology will be studied. Aged-matched normal subjects will be studied in the same manner. Ten patients in each category have already been defined and will serve as the basic patient population. New patients coming to clinic for evaluation will be brought into the program as part of their clinical evaluation.

Patients are seen in the Hypertension-Endocrine Branch Outpatient Clinic and the following clinical studies are performed on an outpatient basis: chest x-ray, EKG, IVP, urinalysis, chemical analysis of blood for Hct, Hgb, CBC, electrolytes, creatinine, cholesterol, triglycerides (after 12 hr fast), plasma renin activity, plasma aldosterone and plasma cortisol (60 ml of blood). Two 24-hour urine specimens are collected for VMA and catecholamines, aldosterone and 17-hydroxycorticosteroids. The patients are

taught to measure their own blood pressure and are asked to monitor it four times a day for two weeks. Normal volunteers are subjected to the same protocol, except for the IVP.

Patients with hypertension who have previously been classified as sodium-sensitive or non-sodium-sensitive are asked to stop medications two weeks prior to admission. Blood pressure is monitored 4 times a day by the patient. If the pressure exceeds 170/110 more than once a day, the patient is admitted immediately.

Patients are admitted to the Clinical Center for a three-week period, placed on metabolic diet of 9 mEq Na, urine collected in 24 hour pools, blood pressure monitored every 4 hours, and every half-half each Saturday (on the Arteriosonde). Blood for Na, K, Cl, CO_2 is drawn on days, 2, 9, 16, and 20. Blood (120 ml) for adrenergic receptors is drawn on day 2. Blood for catecholamines and bradykinin is drawn on two days at the time of peak and nadir blood pressure during the period of high-salt intake.

Furosemide orally (40 mg x 3) is given on day 16. Sodium (240 mEq/d, 6 - 10 mEq tablets four times a day) is added days 9 through 15.

Major Findings:
Preliminary data:

The average mean blood pressure on low-sodium and high-sodium diets: the mean increment of MBP between low- and high-sodium diets differs significantly between "salt-sensitive" and "non-salt-sensitive groups".

The urinary sodium on the seventh day of the high-sodium diet: the urinary sodium on the seventh day of the high-sodium diet is significantly greater in "non-salt-sensitive" patients than that in "salt-sensitive" patients.

Echocardiography: the increment of cardiac output on the high-sodium diet is greater in "salt-sensitive" patients than that in "non-salt-sensitive" patients.

Plasma renin activity and urinary aldosterone: there is no significant difference between "salt-sensitive" and non-salt-sensitive" patients.

Significance:

Salt-sensitive patients cannot excrete sodium "loads" easily. The increment of retained sodium, via an increase in cardiac output, produces the rise of blood pressure. The mechanism by which dietary sodium influence blood pressure may be due to the response of autonomic nervous system to salt loads. The findings in this study may lead to a new classification of hypertensive subjects by physiologic differences in cardiac output and the autonomic nervous system

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-01819-01 HE

PERIOD COVERED
July 1, 1976 to Sept.30, 1977

TITLE OF PROJECT (80 characters or less)

Importance of the Glutathione-S-Transferases in the Detoxification of Acetaminophen

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

P.I.	D.E. Rollins	Research Associate	SCPM DIR NHLBI
	A.R. Buckpitt	Guest Worker	SCPM DIR NHLBI
	J.R. Mitchell	Chief, Section on Clinical Pharm. and Metabolism	SCPM DIR NHLBI

COOPERATING UNITS (if any)

Dr. Rollins is a Research Associate in the Pharmacology-Toxicology Program, NIGMS. Dr. Buckpitt holds an NIH Research Fellowship in NHLBI.

LAB/BRANCH
Office of the Director of Intramural Research

SECTION
Clinical Pharmacology and Metabolism

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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)

The effects of the glutathione transferases on 1) conjugate production and 2) covalent binding of acetaminophen were assessed in vitro. The addition of the glutathione transferases to microsomal incubations containing acetaminophen increased the amount of GSH conjugate produced and decreased the amount of covalently bound metabolite when compared to boiled enzyme controls. These transferase effects were more pronounced at low GSH concentrations (0.25 mM) than at high GSH concentrations (2.5 mM). The addition of transferase enzymes to incubations containing either cysteine or N-acetylcysteine in place of glutathione, had little effect on either conjugate production or covalent binding when compared to boiled enzyme controls.

629

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Although the GSH-transferases have been shown to be important in catalyzing the formation of GSH conjugates with inherent electrophiles such as 2,4-dinitrochlorobenzene, little information is available on the role of the transferases in catalyzing the detoxification of electrophiles produced as a result of metabolic activation. These studies were undertaken to determine whether the GSH-S-transferases would catalyze the conjugation of GSH with acetaminophen.

Methods Employed: Mouse liver microsomes were prepared by standard methods and incubated with ^{14}C-acetaminophen. Mouse liver supernatant was chromatographed on a Sephadex G-25 column to separate endogenous GSH from the protein fraction containing GSH-S-transferases. Incubations also contained GSH and a NADPH-generating system. Covalent binding of ^{14}C-acetaminophen to microsomal proteins was determined as described previously (J. Pharmacol. Ext. Ther. 187: 203-210, 1973). Acetaminophen-GSH conjugate production was determined in the supernatant of the incubation by high pressure liquid chromatography as described previously (Buckpitt et al., Ann. Biochem., in press). Covalent binding and conjugate production were measured in the same incubation.

Major Findings: Increasing the amount of GSH-S-transferases added to an incubation resulted in an increase in the acetaminophen-GSH conjugate production and a decrease in covalent binding. The V_{max} for conjugate production was increased by the presence of the glutathione S-transferases. This effect of the GSH-S-transferases was greatest at lower GSH concentrations (0.25 mM). At higher GSH concentrations (2.5 mM) there was less of a transferase effect. At 5.0 mM GSH there was only a small transferase effect. Cysteine and N-acetylcysteine were not good substrates for the GSH S-transferases.

Significance to Biomedical Research and the Programs of the Institute: Acetaminophen hepatotoxicity is becoming an increasing health problem. Our data suggests that the GSH-S-transferases may play an important role in the detoxication of acetaminophen, particulalry when the concentration of GSH in the liver is low (as occurs during acetaminophen metabolism).

Proposed Course of Project: Completed.

Publications: Manuscript in preparation.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-HL-01821-03 HE

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

The study of the nephrogenous cyclic AMP in patients with hypercalciuria and in normal volunteers.

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

PI:	T. Fujita	Visiting Scientist	HE NHLBI
OTHER:	F.C. Bartter	Chief, Hyper.-Endo. Branch	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Steroid and Mineral Metabolism

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

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)

These studies show that the estimation of nephrogenous cyclic AMP reflects rapid changes in parathyroid activity and it can serve as an index of the entire spectrum of parathyroid function.

In addition, nephrogenous cyclic AMP is increased in patients with "renal" hypercalciuria and normal volunteers taking furosemide 80 mg a day for 8 days, who develop secondary hyperparathyroidism. On the other hand, in "absorptive" hypercalciuria, nephrogenous cyclic AMP is normal, showing that the distinguishing features are a normal or partly suppressed parathyroid function.

631

PHS-6040
(Rev. 10-76)

Project Description:

Recent studies have suggested that the measurement of nephrogenous cyclic AMP may provide a more accurate index of parathyroid activity than does the measurement of parathyroid hormone (a still technically inaccurate determination), or that of serum calcium (which may be normal in normocalcemic hyperparathyroidism).

Hypercalciuria may result from: a) an excessive resorption of bone (primary hyperparathyroidism), b) an enhanced intestinal absorption of calcium (sarcoidosis, hypervitaminosis D), c) an impaired renal tubular reabsorption of calcium (Bartter's Syndrome, renal tubular acidosis). The mechanism of idiopathic hypercalciuria has not been established. At present, two theories deserve special consideration: "absorptive" and "renal" hypercalciuria (Pak, C.Y.C. et al., N. Engl. J. Med. 292: 497, 1975.

In order to more clearly define the various factors involved in the production of hypercalciuria, patients with hypercalciuria from various disease processes are evaluated for their parathyroid function.

Methods Employed:

Patients with hypercalciuria showing: a) normocalcemia, b) recurrent nephrolithiasis, c) presence of unequivocal hypercalciuria (greater than 250 mg/24 hr), d) bone disease, and e) unknown causes of hypercalciuria are studied. Patients with Bartter's syndrome, renal tubular acidosis and osteoporosis are included.

Five healthy young men were studied for the effect upon parathyroid function of hypercalciuria resulting from the administration of furosemide.

Patients with hypercalciuria and normal volunteers are admitted to the Clinical Center for a period of 14 days. They are placed on a metabolic diet of 400 mg Ca, 1000 mg P, 100 mEq Na with daily urine and stool collections. They are well hydrated with distilled water and remain recumbent (except for voiding) throughout all procedures. On the third day, the control day for the calcium infusion, blood is drawn for cyclic AMP, Cr, Ca. On day 4 at 9 a.m., following 2 control periods, calcium 4 mg/kg (calcium gluconate in 500 ml 5% dextrose) is infused over a 3-hour period. Four blood samples for serum Na, K, Cl, total protein, calcium, potassium, magnesium, creatinine, plasma iPTH and cyclic AMP and urine collections for sodium, calcium, potassium, magnesium and creatinine and cyclic AMP are obtained before the infusion and during three 30-minute periods during the last 1.5 hours of the infusion. The serum calcium concentration is not expected to rise above 13 mg%.

Young normal volunteers receive furosemide (40 mg orally) twice daily, and supplemental sodium chloride (100 mEq orally) daily, for 8 consecutive days. The amount of potassium lost each day is added to the next day's so that potassium deficiency does not result. Calcium infusion tests as described above are done during the 8th day of treatment. Ten ml blood is obtained for ionized calcium concentration.

Major Findings:

Cyclic AMP in normal volunteers taking furosemide: nephrogenous cyclic AMP in normal volunteers receiving furosemide is significantly higher than that of controls. During calcium infusion, nephrogenous cyclic AMP is decreased to normal range.

Cyclic AMP in osteoporotic patients with normal excretion rate of calcium: nephrogenous cyclic AMP in osteoporotic patients with normal excretion rate of calcium is not significantly different from those of controls.

Cyclic AMP in patients with "renal" hypercalciuria" nephrogenous cyclic AMP in patients with 'renal' hypercalciuria is higher than that of controls. During calcium infusion, nephrogenous cyclic AMP is decreased to normal.

Cyclic AMP in patients with "absorptive" hypercalciuria: nephrogenous cyclic AMP in patients with "absorptive" hypercalciuria is within normal range.

Significance:

These studies show that the estimation of nephrogenous cyclic AMP reflects rapid changes in parathyroid activity and it can serve as an index of the entire spectrum of parathyroid function. In addition, nephrogenous cyclic AMP is increased in patients with "renal" hypercalciuria and normal volunteers receiving furosemide, since they are accompanied with secondary hyperparathyroidism. On the other hand, nephrogenous cyclic AMP in "absorptive" hypercalciuria is normal, showing that the distinguishing features are a normal or partly suppressed parathyroid function.

Proposed Course:

Additional patients and normal subjects will be studied.

Publications:

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01900-04 HE

PERIOD COVERED

July 1, 1976 to June 30, 1977

TITLE OF PROJECT (80 characters or less)

Prostaglandins in Renal and Vascular Physiology

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

PI:	Robert E. Bowden, M.D.		Staff Associate	HE NHLBI
OTHER:	Harry R. Keiser, M.D.		Deputy Chief	HE NHLBI
	David Horwitz, M.D.		Senior Investigator	HE NHLBI
	John J. Pisano, Ph.D.	Head,	Sec. on Physiol. Chem.	CH NHLBI
	Lauren M. Cagen, Ph.D.		Staff Fellow	CH NHLBI
	John R. Gill, Jr., M.D.		Senior Investigator	HE NHLBI
	Addison A. Taylor, M.D.		Senior Investigator	HE NHLBI
	Robert P. Kimberly, M.D.		Clinical Associate	A&R A
	Paul H. Plotz, M.D.		Senior Investigator	A&R A
	Teruhiro Nakada, M.D.		Guest Worker	HE NHLBI
	Joseph Vinci, M.D.		Staff Associate	HE NHLBI
	Randall Zusman, M.D.		Clinical Associate	HE NHLBI

COOPERATING UNITS (if any)

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Arthritis and Rheumatism Branch

LAB/BRANCH

Hypertension-Endocrine Branch

SE

Section on Experimental Therapeutics

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.5	3.0	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 role of prostaglandins in renal physiology remains unclear. We have shown previously that there is a circadian rhythm for urinary prostaglandin E (iPGE) and that there is no effect of alteration of dietary sodium and potassium on iPGE excretion. In the last year we have demonstrated that alteration of fluid intake, administration of fludrocortisone, a sodium retaining steroid, human serum albumin and adrenal cortical trophic hormone also have no consistent effect on urinary iPGE excretion. These manipulations suggest that prostaglandin E excretion may be independent of sodium retaining steroids. Aspirin administration to patients with systemic lupus erythematosis causes significant falls in renal blood flow and glomerular filtration rate which correlate significantly with the fall in iPGE. Similar findings have been observed with other non steroidal anti-inflamatory agents. Urinary iPGE excretion is elevated in patients with Bartter's syndrome. Treatment of these patients with prostaglandin synthetase inhibitors reverses the abnormalities of the syndrome, including hyperreninemia and hypokalemia proportional to the degree of inhibition of prostaglandin synthesis. The exact mechanism of action of prostaglandins in this disorder remains unexplained.

634

PHS-6040
(Rev. 10-76)

Objectives: 1) Further development of methodology for measuring prostaglandins (PG) in subnanogram range in biologic fluids, and 2) application of these methods to the study of the role of prostaglandins in renal physiology and in the pathology of hypertension.

Methods: Radioimmunoassays have been developed as reported previously by this laboratory, which allow reliable measurements of PGA and PGE with a sensitivity of 150 to 200 pg.

Major Findings: A. Humans

1. iPGE excretion in normal subjects. The range of urinary excretion of immunoreactive prostaglandin E (iPGE) is 30 ± 10 ng/hr (mean ± SD) for normal subjects eating a 109-mEq-sodium and 100-mEq-potassium diet.

a) Administration of fludrocortisone to seven normal subjects eating 109-mEq-sodium diet had no effect on iPGE either acutely or after the period of sodium retention had occurred.

b) Administration of adrenal cortical trophic hormone for two days to 11 subjects while the subjects were eating a 9-mEq-sodium diet had no effect on iPGE excretion, even though there was a marked rise in aldosterone excretion.

c) Reduction of dietary fluid intake from 2500 to 600 ml. daily had no significant effect on iPGE excretion. Plasma osmolality rose significantly from 282 ± 5 mOsm/L to 296 ± 7 as did urinary osmolality from 353 ± 20 to 1115 ± 100 mOsm/L.

d) Infusion of 100 grams/day of human serum albumin for four days to seven subjects caused a consistent increase in body weight, sodium and water retention and a decrease in hematocrit. There were variable changes in creatinine clearance and iPGE excretion. Treatment with indomethacin in one subject followed by albumin infusion produced the same effects on weight gain and sodium or water retention as those produced by albumin alone. iPGE excretion was suppressed by indomethacin and creatinine clearance rose with albumin infusion, with or without indomethacin in this subject.

2. iPGE excretion in hypertensive subjects. Fifteen women with essential hypertension have been studied on a controlled 109-mEq-sodium and 100-mEq-potassium diet. There is no significant difference in iPGE excretion between these women and the normal subjects. Four patients were also studied on a 9-mEq-sodium diet; no effect on iPGE excretion was noted with the change in sodium intake.

a) In order to further investigate the mechanisms controlling iPGE excretion, three patients with pheochromocytomas were studied under control conditions, with phenoxybenzamine treatment and with alpha-methyl-paratyrosine treatment. There was no change in iPGE excretion with either treatment.

Urinary iPGE excretion is elevated in patients with systemic lupus erythematosis and falls in proportion to the fall in creatinine clearance that occurs when prostaglandin synthetase inhibitors are administered. This fall in creatinine clearance is probably secondary to a decrease in renal blood flow which may be dependent upon prostaglandin synthesis in these patients. The findings of supra-normal levels of urinary iPGE in these patients is as yet unexplained, but may be secondary to the effect of the disease on the kidney.

The elevation of urinary iPGE excretion in patients with Bartter's syndrome may explain the pathophysiology of the syndrome. The degree of the correction of the chemical abnormalities of the syndrome with prostaglandin synthetase inhibitors probably depends on the degree of suppression of prostaglandin production in the kidney. Serum potassium and plasma renin activity, in particular, change more with greater inhibition of prostaglandin synthesis. Prostaglandin synthetase inhibitors also have a marked effect on creatinine clearance in patients with the syndrome. Long term therapy may also require normalization of prostaglandin E excretion, or the combination of a prostaglandin synthetase inhibitor and an aldosterone antagonist to maintain a normal serum potassium.

Proposed Course of Project: 1) Study of prostaglandin excretion in patients with Bartter's syndrome after long term therapy with prostaglandin synthetase inhibitors.

2) Study further the relationship of prostaglandins and the renin-angiotensin-aldosterone system.

3) Study of prostaglandin E excretion in patients and normals in whom the renin-angiotensin system is either stimulated or inhibited by either pharmacologic or physiologic means.

4) Study the effect of prostaglandin synthetase inhibitors on creatinine clearance in patients with systemic lupus erythematosis and other collagen vascular diseases.

5) Study prostaglandin E excretion in normal and hypertensive subjects to learn more about the control mechanisms for the renal production of prostaglandins.

Publications:

1. Gill, J.R., Jr., Frolich, J.C., Bowden, R.E., Taylor, A.A., Keiser, H.R., Seyberth, H.W., Oates, J.A., and Bartter, R.D.: Bartter's syndrome: A disorder characterized by high urinary prostaglandins and a dependence of hyperreninemia on prostaglandin synthesis. Am. J. Med. 61: 43-51, July 1976.

2. Bartter, F.L., Gill, J.R., Frolich, J.C., Bowden, E.R., et al.: Prostaglandins are overproduced by the kidneys and mediate hyperreninensis in Bartter's syndrome. Trans. Assoc. Am. Phys. 89: 77-81, 1976.

3. Cagen, L.M., Fales, H.M., Bowden, R.E.: Formation of Glutathione Conjugates of Prostaglandin A_1 in human erythrocytes. Prostaglandins in Hematology Ed. by Silver, M.J. and Smith, J.B. Spectrum Publications, New York, 1976.

4. Bowden, R.E., Ware, J.H., DeMets, D.L. and Keiser, H.R. Urinary excretion of immunoreactive prostaglandin E: A circadian rhythm and the effect of posture. Prostaglandins, 1977, in press.

5. Bowden, R.E., Gill, J.R., Radfar, W. et al. Effect of different Prostaglandin synthetase inhibitors on immunoreactive prostaglandin excretion in Bartter's syndrome. JAMA, 1977, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01903-03 HE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Plasma Prekallikrein and Kininogen 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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	J.M. Vinci, M.D.	Staff Associate	HE NHLBI
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	H.R. Keiser	Deputy Chief	HE NHLBI

COOPERATING UNITS (if any)
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Arthritis and Rheumatism Branch, NIAMDD

LAB/BRANCH
Hypertension-Endocrine Branch

Sc
Section on Experimental Therapeutics

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.25	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)

Prekallikrein (PPK) and total kininogen (KGN) were measured in human plasma. PPK was determined by radiochemical esterolytic assay, KGN by radioimmunoassay as bradykinin after trypsin digestion of plasma. In normal volunteers, no changes in PPK or KGN were effected by variation of dietary potassium, administration of fludrocortisone, or dehydration. In hypertensives, no variation in PPK or KGN was observed with manipulation of dietary sodium, administration of indomethacin, or administration of SQ 20,881. In patients with systemic lupus erythematosis, aspirin therapy did not change PPK, but did effect a small decrease in KGN.

PHS-6040

Objectives: Although we have demonstrated elevated urinary kallikrein (HUK) excretion in states of excess sodium retaining steroid activity and reduced HUK in certain patients with essential hypertension, the role of the plasma kallikrein kinin system (KKS) remains unclear. We conducted several studies on normal volunteers and with hypertension in an attempt to elucidate the function of the KKS. Because of reports of normalization of elevated kininogen in patients with rheumatoid arthritis treated with indomethacin, we also studied several patients with systemic lupus erythematosis before and during aspirin treatment.

Methods: We determined values of plasma prekallikrein (PPK) and total plasma kininogens (KGN) by methods previously described (Annual Report No. Z01 HL 01903-02 HE). Values for PPK are expressed in tosyl-l-arginine-methyl ester (TAMe) units (TU) per ml plasma. Values for KGN are expressed in ug bradykinin (BK) generated per ml plasma.

Several studies were undertaken with normal volunteers after informed consent was obtained. Dietary sodium intake was varied from 9 mEq to 109 mEq to 259 mEq per day. Dietary potassium intake was varied from 25 mEq to 185 mEq per day. We administered fludrocortisone 0.5 mg/day to normal volunteers to expand the extracellular fluid volume. Mild dehydration of normal subjects was achieved by water restriction to 600 ml per day for 4 days.

Five hypertensive subjects were studied on normal (109 mEq) and low (9 mEq) sodium diets with and without administration of indomethacin, 75 /day for 7 days. Ten hypertensive subjects on 109 mEq or 9 mEq were also studied during administration of SQ 20,881 (3 mg/kg as a single bolus). This inhibitor of the angiotensin converting enzyme/kininase system is discussed in more detail in Annual Report #Z01 HL 01905-07 HE. Arterial blood samples were obtained immediately before injection of the SQ 20,881 bolus, and at 15, 60, 120 and 240 minutes thereafter.

Nine patients with active systemic lupus erythematosis (SLE) nephritis were also evaluated before, during, and after therapy with aspirin. Duration of therapy ranged from 5 to 21 days with blood salicylate levels monitored daily. Samples for PPK and KGN were obtained every 1 to 3 days and mean values for control and treatment periods were compared.

Results: (values expressed as $\bar{x} \pm$ SEM)

A. Normal volunteer studies

1. Variation of dietary sodium. Five additional volunteers demonstrated no significant change in PPK or KGN as a function of variation of dietary sodium from 9 mEq to 109 mEq to 259 mEq. (This supports findings previously reported in 3 subjects.) Plasma renin activity (PRA) and urinary aldosterone excretion (AER) changed as expected.

22. Variation of dietary potassium. No change in PPK or KGN was observed when volunteers were changed from 185 mEq to 25 mEq potassium diets (n=5), despite appropriate changes in PRA and AER.

3. Fludrocortisone: After 7 days of fludrocortisone administration to 8 volunteers, no change in PPK or KGN was observed.

4. Dehydration: After 4 days of 600 cc fluid restriction in 4 subjects, PPK remained unchanged (1.14 I0.06 vs. 1.17 ± 0.05 TU/ml.) and KGN values increased from 2.44 I0.10 to 2.96 ± 0.17 µg BK/ml. Average weight loss during the study was approximately 3% body weight.

B. Hypertensives

1. Variation in dietary sodium intake from 109 mEq to 9 mEq had no effect on PPK or KGN (n=10).

2. Indomethacin had no effect on PPK or KGN in hypertensives (n=5).

3. SQ 20,881 did not effect any change. in PPK or KGN (n=9).

C. SLE

1. Aspirin therapy caused no changes in PPK (n=9).

2. There was a small decrease in KGN on aspirin therapy (4.96 ± 0.38 to 4.56 ± 0.24, n=4) which was not statistically significant.

Significance: It is difficult to implicate PPK and KGN in any physiologic control mechanism related to hypertension. Negative results with various experimental modalities over the past two years (dietary manipulations, amine infusions, angiotensin converting enzyme blockade, indomethacin administration) demonstrate the limited usefulness of isolated PPK and KGN determinations. The broad range of values from PPK (0.7 to 2.2 TU/ml; mean ± 2 S.D.=1.3 ± 0.5 TU/ml) and KGN (2.3 to 6.5 µg BK/ml; mean ± 2 S.D.=4.0 ± 1.6 µgBK/ml) in normal subjects further limits their usefulness.

PPK and KGN may still serve as a useful benchmark for overall evaluation of the plasma kallikrein-kinin system when combined with measurements of plasma bradykinin and angiotensin converting enzyme (kininase II).

Proposed Course of Project: A collaborative effort in overall characterization of the kallikrein-kinin system will continue within the Experimental Therapeutics Section.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01905-07 HE

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Urinary and Plasma Kallikrein and Kinin

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

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OTHER:	D. Horwitz, M.D.	Senior Investigator	HE NHLBI
	H.R. Keiser, M.D.	Deputy Chief	HE NHLBI
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	P.J. Cannon, M.D.	Columbia University	

COOPERATING UNITS (if any)

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Department of Medicine, Columbia University

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Experimental Therapeutics

INSTITUTE AND LOCATION

NHLBI, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	2	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)

Urinary kallikrein and kinin and plasma bradykinin were measured in normal subjects and in patients with hypertension, Bartter's syndrome, and scleroderma on controlled intakes of sodium, potassium and fluid. Plasma bradykinin in normal subjects is responsive to changes is posture and sodium intake and correlates with the level of plasma renin activity, but not to the level of sodium-retaining steroid. Urinary kallikrein increases with the level of sodium retaining steroid and is highly correlated with aldosterone excretion in all studies but urinary kinin excretion is not affected by changes in urinary kallikrein or the level of sodium retaining steroids. Prostaglandin synthetase inhibition decreased urinary kallikrein but had no effect on plasma or urinary kinins. Large changes in ECF volume had no effect on plasma or urinary kinins. Patients with Bartter's syndrome have significantly elevated basal urinary kallikrein and plasma bradykinin but subnormal urinary kinin excretion. During prostaglandin synthesis inhibition urinary kallikrein and plasma bradykinin decreased while urinary kinin excretion increased toward normal. These data indicate an involvement of the kallikrein-kinin system in this syndrome and its relationship to other vasoactive systems.

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Project Description: To study the role of the plasma and urinary kallikrein-kinin system and its relationships to the renin-angiotensin and prostaglandin systems in normal subjects, in patients with hypertension or renal disease and in animals.

Methods: Urinary Kallikrein is measured with a previously described radiochemical assay [NHLI-79(c)].

Urinary kinin is measured by a radioimmunoassay developed in this lab with an antibody that binds bradykinin, kallidin and methionyl-lysl-bradykinin, and thus gives a measure of total immunoreactive kinins.

Plasma bradykinin is processed for radioimmunoassay by a modified method of Talamo and Spragg.

Sheep plasma containing ^{125}I-tyr-8 bradykinin and its products of hydrolysis were separated by CM-sephadex (C-25) chromatography.

Urinary kallikrein and plasma and urinary kinins were measured in normal subjects and in patients with hypertension, Bartter's Syndrome, and scleroderma. All studies were performed on a controlled intake of sodium and potassium. We studied the effects of sodium and potassium intake, fluorocortisone (0.5 mg/d for 6 days), ACTH (80 units/d for 2 days), indomethacin (150 mg/d for 3 days), dehydration (600-800 ml total fluid intake for 3 days) and albumin infusion (400 gms/d for 4 days) on the kallikrein-kinin system in normal man to determine the relationship of this system to the level of sodium-retaining steroid activity, plasma renin activity, prostaglandins and fluid balance. Patients with Bartter's syndrome were studied on controlled diets before and during indomethacin. Patients with hypertension were characterized on controlled diets (109 mEq and 9 mEq/d sodium); the angiotension-converting enzyme inhibitor, SQ20881 (1-3 mg/kg) was given to 13 patients. Twenty patients with scleroderma were characterized on diets containing 109 mEq/d sodium.

Twelve anesthetized sheep (sodium pentobarbital) were given 50 µCi boli ^{125}I-tyr-8-BK before and after SQ20881 (6 mg/kg) and A_I (25-50 ng/kg/min); samples were obtained after 1 pulmonary passage for measurement of ^{125}I-tyr-8 bradykinin and its products of hydrolysis to determine the inhibitory effects of these peptides on the hydrolysis of bradykinin by angiotensin converting enzyme.

Major Findings:

1) Normal Man: In 29 normal subjects fed 109 mEq/d sodium and 100 mEq/d potassium mean kallikrein excretion was 8.7 ± 0.7 TU/d (mean ± SEM), mean kinin excretion was 13.4 ± 0.9 µg/d and mean plasma bradykinin was 3.3 ± 0.3 ng/ml (subject recumbent) and 4.6 ± 0.5 ng/ml (subject upright). The rise in plasma bradykinin with upright posture is significant ($p<0.002$. Seventeen normal subjects fed 9 mEq/d sodium had significantly elevated urinary kallikrein, 18.4 ± 1.4 TU/d ($p<0.001$), plasma bradykinin (subject recumbent), 5.4 ± 0.7 ng/ml ($p<0.001$) and 8.3 ± 0.7 ng/ml (subject upright) ($p<0.001$). Urinary kinin excretion was not significantly different (12.5 ± 1.3 µg/d). In these studies plasma renin activity was highly correlated with the level of plasma bradykinin ($r=0.88$, $p<0.001$).

In 5 subjects fed 185 mEq/d and then 25 mEq/d potassium, mean Kallikrein excretion decreased from 14.7 ± 1.9 TU/d to 4.8 ± 0.7 TU/d ($p=0.013$) and aldosterone excretion decreased from 18.0 ± 3.1 µg/d to 2.4 ± 0.5 µg/d. Mean kinin excretion was not significantly affected (11.4 ± 3.0 to 11.5 ± 3.1 µg/d) nor was plasma bradykinin (3.2 ± 1.0 to 3.2 ± 1.1 ng/ml). When subjects were upright plasma bradykinin decreased from 4.6 ± 1.8 to 3.6 ± 1.8 ng/ml ($p=0.05$).

In 9 subjects given fludrocortisone while they were fed 109 mEq/d sodium, mean kallikrein excretion increased from 10.4 ± 1.2 TU/d to 23.6 ± 4.3 TU/d ($p=0.004$) but neither urinary nor plasma kinins were increased.

The administration of ACTH to 11 sodium-depleted subjects increased urinary kallikrein from 20.5 ± 1.9 TU/d to 34.7 ± 3.3 TU/d ($p<0.001$) after 48 hours, but had no effect on plasma or urinary kinins. Thus, kallikrein excretion is dependent on the level of sodium-retaining steroid (over an 80-fold range in Aldosterone and a 30-fold range in kallikrein excretion, kallikrein and aldosterone excretion rates are highly correlated, $r=0.92$, $p<0.001$) but plasma and urinary kinins are not. Urinary kinins are unrelated to either kallikrein excretion, the level of sodium-retaining steroid or plasma renin activity.

The administration of indomethacin to 6 sodium-depleted normal subjects decreased kallikrein excretion from 25.4 ± 5.1 TU/d to 15.8 ± 4.0 TU/d ($p<0.02$) and from 43.1 ± 6.0 TU/d to 24.1 ± 3.5 TU/d ($p<0.001$) during concomitant ACTH. Neither plasma nor urinary kinins were affected by indomethacin indicating that the effects of indomethacin in patients with Bartter's syndrome (vide infra) may reflect a fundamental derangement in kinin physiology in these patients.

In 10 normal subjects fed diets containing 69 mEq/d sodium, 70 mEq/d potassium and 2500 ml/d TOTAL fluid followed by 600-800 ml/d total fluid, mean kallikrein excretion decreased 20.0 ± 3.8 TU/d to 14.2 ± 2.7 TU/d ($p=0.006$) as aldosterone decreased from 25.5 ± 4.4 to 14.1 ± 1.9 ng/d ($p=0.006$); urine volume decreased from 1809 ± 97 ml/d to 593 ± 21 ml/d ($p<0.001$) and urinary osmolarity increased from 382 ± 24 to 1223 ± 69 mOSm/L ($p<0.001$). Neither urinary nor plasma kinins were affected.

In seven normal subjects fed 259 mEq/d sodium and 100 mEq/d potassium and infused with albumin, sodium excretion decreased from 236 ± 8 to 150 ± 6 mEq/d ($p<0.001$), aldosterone decreased from 5.9 ± 0.9 to 2.1 ± 0.3 µg/d ($p<0.001$), creatinine clearance increased from 118 ±7 to 148 ± 15 cc/min ($p=0.008$), kallikrein excretion decreased from 6.8 ± 1.3 TU/d to 4.1 ± 0.7 TU/d ($p<0.001$), but urinary kinins were not affected. Plasma bradykinin (subject recumbent) was also not affected (2.9 ± 0.7 to 2.4 ± 0.6 ng/ml). Thus acute changes in extracellular fluid volume have no effect on plasma and urinary kinins.

2) Patients with Bartter's Syndrome: Seven patients with Bartter's Syndrome fed 109 mEq/d sodium and 270 mEq/d potassium had significantly elevated basal urinary kallikrein (24.8 ± 3.2 TU/d) compared to normal subjects fed 109 mEq/d sodium (812 ± 7.4 ± TU/d (mean ± 2 SD), $p<0.001$), elevated plasma bradykinin (13.2 ± 4.2 ng/ml) compared to normal subjects (3.0 ± 1.8 ng/ml (mean ± 2 SD), $p<0.001$), but subnormal kinin excretion (4.3 ± 1.2 µg/d) compared to normal subjects (13.3 ± 8.7 µg/d (mean ± 2 SD), $p<0.001$). During prostaglandin synthetase inhibition with either indomethacin or ibuprofen, urinary kallikrein

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decreased to 12.4 ± 2.0 TU/d ($p<0.005$), plasma bradykinin decreased to 3.9 ± 0.9 ng/ml ($p<0.05$) and kinin excretion increased to 8.5 ± 2.5 µg/d ($p<0.005$). With prostaglandin inhibition, there was sodium and potassium retention, correction of hypokalemia and a decrease in plasma renin activity and aldosterone excretion. These data indicate an involvement of the kallikrein-kinin system in Bartter's syndrome and suggest a fundamental derangement of kinin physiology in these patients.

3) Hypertensives:

A. Comparison study - see Project Report Z01 HL 01911-01 HE

B. Of thirteen hypertensive patients (mean diastolic blood pressure was 95 ± 3 torr) who received 1-3 mg/kg SO20881 when fed diets containing 109 mg/d sodium, 10 subjects showed biochemical evidence of converting enzyme inhibition by a sustained reduction in the level of angiotensin II (52 ± 11 to 29 ± 5 pg/ml blood, $p<0.005$) over a 2 hour study period. In these subjects, diasolic blood pressure (subject recumbent) decreased by 7 ± 2 mm Hg ($p<0.02$) and paralleled the fall in angiotensin II. The level of bradykinin was not affected (7.0 ± 1.8 to 5.5 ± 1.3 ng/ml) but plasma immunoreactive prostaglandin E increased from 149 ± 22 to 268 ± 47 pg/ml ($p<0.01$) during the study period. In 9 patients fed 9 mEq/d sodium diastolic blood pressure fell by 7-10 mm Hg ($p=0.02$) during the study but neither angiotensin II, bradykinin nor prostaglandin E were significantly affected in this group.

4) Scleroderma: Twenty subjects with different stages of renal scleroderma showed a significant correlation between kinin excretion and creatinine clearance ($p<0.001$). Kallikrein excretion was not correlated with creatinine clearance.

5) Sheep: Preliminary results in six sheep given SO20881 (6 mg/kg) showed a 50% ± ($p<0.001$) reduction in the products of ^{125}I-tyrosine-8-bradykinin hydrolysis after 1 passage through the lung but no difference after recirculation. This indicates that although the hydrolysis of bradykinin by angiotensin-converting enzyme can be significantly decreased by SO20881, hydrolysis by plasma kininase continues during recirculation. Thus SO20881, probably has no lasting effect on the level of bradykinin.

Preliminary results in six sheep given injections of angiotensin I (25-50 ng/kg/min); no effect on the hydrolysis of ^{125}I-tyrosine-8-Bk. Thus, the competition inhibition of AI for converting enzyme does not contribute to the elevations in plasma bradykinin in hyper-reninemic states.

Significance to Biomedical Research and the Program of the Institute:
Originally we had presumed that urinary kinins would parallel changes in urinary kallikrein. The data obtained this year indicate that there is no correlation between urinary kallikrein and kinin. In fact it appears that normal changes in salt and water metabolism by the kidney produce no change in urinary kinins.

The plasma kallikrein-kinin system is entirely separate from that in the blood. The new data indicate that plasma bradykinin correlates well with plasma renin activity and suggests that the former may serve to modulate the vasoconstriction of the latter. This would be an important interaction in the regulation of blood pressure.

Finding that the plasma and urinary kallikrein-kinin systems are altered in patients with Bartter's Syndrome adds additional information about the interactions with the renin-angiotensin system. It also adds another possible explanation for why these patients are normotensive inspite of high levels of renin, angiotensin and aldosterone.

Proposed Course of Project:

1. Study possible role of urokininogen as a determining factor in excretion of urinary kinin.
2. Study effects of renal disease on levels of urinary kininogen, kallikrein and kinin.
3. Study of urinary kininase activity to determine if it is an important factor in regulation of kinin excretion.
4. Study regulation of plasma bradykinin and role of angiotensin-converting enzyme in physiologic control of this potent vasodilator.

Pubs.:

1. Hial, V., Keiser, H.R., and Pisano, J.J. Origin and content of methionyl-lysyl-bradykinin, lysyl-bradykinin and bradykinin in human urine. Biochem. Pharmacol. 25: 2499-2503, 1976.
2. Zinner, S.H., Margolius, H.S., Posner, B., Keiser, H.R., Kass, E.H. Familial aggregation of urinary kallikrein concentration in childhood: relation to blood pressure, race and urinary electrolytes. Amer. J. Epidem. 104: 124-132, 1976.
3. Keiser, H.R., Margolius, H.S., Brown, R., Rhamey, R., and Foster, J. Urinary kallikrein in patients with renovascular hypertension. (Ed.: Pisano, J.J. and Austen, K.F.) Chemistry and Biology of the Kallikrein-Kinin System in Health and Science. Washington, D.C., U.S.G. P.O., 1976, pp.423-426.
4. Newball, H.H., Keiser, H.R., Webster, M.E., Pisano, J.J. Effects of bradykinin on hyman airways. ibid, pp.505-511.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01907-02 HE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Catecholamine Metabolites in Normal and Hypertensive Subjects

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

PI:	J.L. Izzo, M.D.	Staff Associate	HE NHLBI
OTHER:	D. Horwitz, M.D.	Senior Investigator	HE NHLBI
	J.J. Pisano, Ph.D.	Head, Section on Physiological Chemistry	CH NHLBI
	H. Fales, Ph.D.	Chief, Laboratory of Chemistry	CH NHLBI
	H.R. Keiser, M.D.	Deputy Chief	HE NHLBI

COOPERATING UNITS (if any)

Laboratory of Chemistry, NHLBI

LAB/BRANCH
Hypertension-Endocrine Branch

SE
Section on Experimental Therapeutics

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	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)

Gas chromatography with electron-capture detection has been used to measure total methoxyhydroxyphenyl glycol (MHPG) in human urine. Previous methods employing flame-ionization detection have proven insufficiently sensitive for our purposes. Normotensive and hypertensive subjects are under study for differences in MHPG excretion. The antihypertensive drugs clonidine, propranolol, and guanethidine are also being tested for their effects on MHPG excretion. The relationship of sodium intake and extracellular fluid volume (changed by dehydration or albumin infusion) to MHPG excretion in normal volunteers is also under evaluation. New methodology for determination of the sulfate conjugate of MHPG (believed to originate predominately in the central nervous system) via paired-ion chromatography is under development.

PHS-6040
(Rev. 10-76)

Objectives: 1) To evaluate methoxyhydroxyphenyl glycol (MHPG) excretion as a possible reflector of sympathetic nervous system (SNS) activity.

2) To evaluate the role of the SNS in essential hypertension.

3) To measure neurotransmitters and their metabolites as mediators of physiologic and pathologic phenomena.

Methods: 1) Storage - 24 hour urine samples are refrigerated during collection, aliquotted, and stored frozen at -4°C in polypropylene vials.

2) Assay - Buffered urine samples are incubated with glusulase (aryl sulfatase - β glucuronidase from Helix pomatia) for 20-24 hours at pH 6.5, extracted with ethyl acetate, dried under a nitrogen stream, stored in a vacuum overnight, derivatized with trifluoroacetic anhydride (TFAA) and gas chromatographed with electron-capture (EC) detection. Each sample is assayed in quadruplicate and quantitated via addition of internal standard to two of the samples.

3) Clinical Studies

a) Comparison of untreated hypertensives with normotensives. Twenty-two pairs of subjects on controlled diets were matched for age, sex, and race, and studied on 109 mEq and 9 mEq daily sodium intakes.

b) Drug studies. Hypertensive subjects, off medications for at least two weeks, are hospitalized on controlled diets for trials of various antihypertensive agents. Daily urine samples and multiple blood pressure readings are obtained on each patient.

c) Normal volunteer studies. Informed consent was obtained for the effect of changes in dietary sodium (9,109 and 259 mEq/n=6) on MHPG. Extracellular fluid volume variation was achieved through mild dehydration (600 ml fluid restriction for four days in 8 subjects) and combination high salt (259 mEq Na^+) intake with albumin infusion (n=8).

Major Findings: 1) Storage - Samples stored as indicated are stable for at least 9 months.

2) Methodology - Considerable difficulty was encountered in the use of present methods to give consistently reproducible results. Initially flame-ionization detection proved insufficiently sensitive for use with reasonable volumes of urine in large numbers of samples. Problems of non-linearity of EC detectors are well known, especially when working near the upper limits of sensitivity of the system, here dictated by relatively high levels of background. Stability of trifluoroacetic esters has been enhanced through rigid adherence to anhydrous conditions. Internal standards using commercially available MHPG as piperazine salt have been found by GC-mass spectroscopy to

undergo polymerization when exposed to heat, light, and acid conditions. Nevertheless, streamlining of methodology (with elimination of unnecessary wash and extraction steps) and an increase in the amount of urine assayed has overcome many problems and led to a workable method.

3) Clinical Studies - Methodological difficulties have retarded MHPG data aquisition on the several studies already completed.

a) Comparison of matched hypertensive and normotensive subjects (age 19-63, n=8). MHPG excretion in normals is 2.40 ± 0.39 mg/day (mean ± SEM) compared to 3.04 ± 1.07 mg/day for hypertensives. Blood pressure difference in these groups is approximately 20/15 mm Hg. Variation of dietary Na^+ from 109 mEq to 9 mEq daily had no effect on MHPG (n=6) in these subjects. Fourteen more pairs are being evaluated.

b) Drug studies. Three patients have been given clonidine 0.3 to 0.8 mg/day with approximately 50% reduction in MHPG excretion paralleling 20-30 mm reductions in blood pressure. One patient each on proprandol (30 mg initially, increased to 220 mg/day over 8 days), guanethidine (25 mg initially, increased to 120 mg over 11 days), and l'ergotrile (a dopaminergic agonist used in Parkinsonism) each demonstrated a 50% or greater reduction in MHPG excretion on therapy, as mean pressures fell 15 to 20 mm Hg.

The diverse actions of these drugs suggests that there is probably a large peripheral component to the total MHPG determination employed here. Significance of these findings remains to be determined.

c) Normal volunteer studies. One subject each on dehydration and albumin infusion demonstrated a 100% increase in baseline MHPG excretion. Further studies are pending.

d) New methodology. Paired-ion chromatography works well for separation of pure MHPG sulfate. Application to urine samples is underway.

Significance to Biomedical Research and the Program of the Institute:
Considerable controversy still exists over the role of the sympathetic nervous system in development and maintenance of essential hypertension. Several lines of evidence suggest that MHPG is the primary metabolite of norepinephrine (NE) in the central nervous system (CNS). Urinary MHPG excretion may therefore be an indicator of CNS NE metabolism. Peripheral NE metabolism also contributes to total MHPG excretion, however. Urinary MHPG is excreted as both glucuronide and sulfate conjugates in roughly equal proportions. It is probable that liver conjugation results predominately in glucuronidation, while CNS conjugation results in sulfation. Present methods evaluate total MHPG excretion; it would be obviously desirable to be able to measure MHPG-sulfate alone.

It is hoped that MHPG excretion may serve as a useful marker of overall sympathetic nervous activity. Such a marker would be invaluable not only to the study of essential hypertension, but to many related problems as well.

Proposed Course of Project: 1) Continue the present studies of normotensive and hypertensives subjects as outlined in this report.

2) Evaluate other drugs for effects on MHPG.

3) Evaluate the role of psychological and physiological stress on MHPG.

4) Continue to develop the selective assay for MHPG sulfate.

5) Revise and extend assay procedures for other catecholamine metabolites, especially homrovanillic acid.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01908-02 HE

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Prostaglandin Biosynthesis and the Role of Prostaglandins in Cardiovascular Physiology

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

PI:	R.M. Zusman	Clinical Associate	HE NHLBI
OTHER:	H.R. Keiser	Deputy Chief	HE NHLBI
	J.M. Vinci	Staff Associate	HE NHLBI
	R.E. Bowden	Staff Associate	HE NHLBI
	J.S. Handler	Chief, Section on Physiology	LKEM NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine Branch

SE

Section on Experimental Therapeutics

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute

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)

Angiotensin II, bradykinin, and vasopressin stimulate PGE_2 biosynthesis in the rabbit renomedullary interstitial cell in tissue culture. Hormonal stimulation of PGE_2 biosynthesis is mediated by phospholipase activation and can be inhibited by mepacrine. Hormonal stimulation of PGE_2 biosynthesis is dependent upon protein synthesis and can be inhibited by inhibitors of protein synthesis, such as cycloheximide. A similar pathway for vasopressin-stimulated PGE biosynthesis was found in the toad urinary bladder where vasopressin-stimulated PGE biosynthesis inhibits vasopressin-stimulated water flow. PGE biosynthesis in the toad urinary bladder is inhibited by the sulfonylureas, which enhance vasopressin-stimulated water flow.

In studies of normal man it was found that ACTH and prostaglandin synthesis inhibition interact to produce inappropriate anti-diuresis by an unknown mechanism.

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PHS-6040
(Rev. 10-76)

Objectives: To study the biosynthesis of prostaglandins and their role in cardiovascular physiology.

Methods: Prostaglandins in biologic fluids were measured via radioimmunoassays developed in this laboratory and described in previous annual reports.

Major Findings: 1) Prostaglandin E_2 Biosynthesis by Rabbit Renomedullary Interstitial cells grown in tissue culture.

In the past year we have extended our previous observations of prostaglandin E_2 (PGE_2) biosynthesis by rabbit renomedullary interstitial cells. Angiotensin II, bradykinin, arginine vasopressin, and hyperosmolality stimulate PGE_2 biosynthesis by stimulating the release of arachidonic acid from the cellular arachidonic acid-storage pool. Potassium and corticosteroids diminish PGE_2 biosynthesis by decreasing arachidonic acid release. Inhibitors of protein synthesis prevent angiotensin II, bradykinin or vasopressin-stimulated arachidonic acid release and PGE_2 biosynthesis.

2) Role of PGE in vasopressin-stimulated water flow in the toad urinary baldder in vitro.

PGE inhibits vasopressin-stimulated water flow in the toad urinary bladder in vitro. We found that vasopressin stimulates arachidonic acid release and PGE biosynthesis in the toad urinary bladder. Vasopressin-stimulated PGE biosynthesis inhibits the water permeability response of the toad bladder. Although theophylline, and cyclic AMP mimic vasopressin in stimulating water flow; they have no effect on PGE biosynthesis. Vasopressin-stimulated arachidonic acid release was blocked by mepacrine, which resulted in lower PGE biosynthesis and enhanced vasopressin-stimulated water flow. Vasopressin-stimulated water flow was enhanced by inhibitors of arachidonic acid cyclo-oxygenase, such as naproxen, indomethacin, meclofenamic acid, ibuprofen, and the sulfonylureas, chlorpropamide, tolbutamide, and glyburide.

3) The effect of prostaglandin synthesis inhibition on adrenal and renal function in normal man.

Prostaglandin synthesis inhibition (PGSI) with indomethacin, 150 mg/day, significantly lowered plasma PGE concentration and urinary PGE excretion but had no effect on basal or ACTH-stimulated plasma cortisol, or urinary 17-hydroxy or 17-keto steroids. PGSI did result in a lower aldosterone excretion rate primarily due to a fall in plasma renin activity. PGSI and ACTH interact to result in marked water retention, with a fall in plasma sodium, plasma osmolality and free water clearance and an increase in urinary osmolality. The mechanism of inappropriate anti-diuresis induced by ACTH and PGSI may be due to the enhanced sensitivity of the kidney to the low levels of anti-diuretic hormone persisting in the circulation.

Significance: These studies have elucidated the pathways responsible for hormonal stimulation of PGE_2 biosynthesis. In both rabbit renomedullary interstitial cells in tissue culture and toad urinary bladders in vitro it was

found that hormones stimulate phospholipase activity and result in arachidonic acid release and PGE biosynthesis. Mepacrine inhibits phospholipase-mediated arachidonic acid release. The stimulation of PGE by hormones is dependent upon protein synthesis, and can be inhibited by inhibitors of protein synthesis, such as cycloheximide.

In studies of renal function in normal man, it was found that ACTH and inhibition of prostaglandin biosynthesis results in inappropriate anti-diuresis. The mechanism of inappropriate anti-diuresis is not known and merits further investigation.

Proposed Course of Project: 1) Studies of the excretion or prostaglandins in patients with various forms of hypertension.

2) Further studies of the interactions of prostaglandins and the kallikrein-kinin system in man and animals.

Publications:

1. Zusman, R.M., and Keiser, H.R.: Prostaglandin biosynthesis by rabbit renomedullary interstitial cells in tissue culture: Stimulation by angiotensin II, bradykinin, and arginine vasopressin. Journal of Clinical Investigation 60: 215-223, 1977.

2. Zusman, R.M., and Keiser, H.R. Prostaglandin E_2 biosynthesis by rabbit renomedullary interstitial cells in tissue culture: Mechanism of stimulation by angiotensin II, bradykinin, and arginine vasopressin. Journal of Biological Chemistry 252: 2069-2071, 1977.

3. Zusman, R.M., Keiser, H.R., and Handler, J.S. Vasopressin-stimulated prostaglandin E biosynthesis in the toad urinary bladder: Effect on water flow. Journal of Clinical Investigation. In press (1977).

4. Zusman, R.M., Keiser, H.R., and Handler, J.S. Inhibition of vasopressin-stimulated prostaglandin E biosynthesis by chlorpropamide in the toad urinary bladder: Mechanism of enhancement of vasopressin-stimulated water flow. Journal of Clinical Investigation. In press (1977).

5. Zusman, R.M., Keiser, H.R., and Handler, J.S. A hypothesis for the molecular mechanism of action of chlorpropamide in the treatment of diabetes mellitus and diabetes insipidus. Fed. Proc. In press (1977).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01909-03 HE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Information Retrieval in Pharmacology

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

PI:	Elise A. Brown, Ph.D.	Research Pharmacologist	HE NHLBI
OTHER:	Harry R. Keiser, M.D.	Deputy Chief	HE NHLBI
	Harriet M. Maling, Ph,D.	Head, Section on Physiology	CP NHLBI

COOPERATING UNITS (if any)

Laboratory of Chemical Pharmacology, NHLBI

LAB/BRANCH
Hypertension-Endocrine Branch

SE
Section on Experimental Therapeutics

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Information retrieval and appraisal activities have been established for the areas of biochemical pharmacology, therapeutics and toxicology. The project aids new research by determining the extent to which a subject has already been explored and by examining the available procedures for answering a question. The computer files of the National Library of Medicine and several commerically available computer files are utilized. Other sources of information are the personal files of the investigator and the informed opinions of various other investigators.

PHS-6040
(Rev. 10-76)

Objectives: The purpose of this project is to facilitate the initiation of laboratory and clinical research projects by determining the present knowledge on a topic and by finding the methods necessary to solve the problem. Patient care physicians need knowledge of drug interactions and other toxocological data for their experimental projects.

Methods: The complex nature of modern medical research requires that both physical and chemical information and clinical information are readily available. Many of the procedures used in research are not standardized but are changed to suit the nature of the problem. A large portion of biomedical information is contained in the Medline, Toxline and other files of the National Library of Medicine. Physical-chemical data and additional biomedical information is obtained from some of the 60 computerized files of the Lockheed Dialog service. There are now three Chemical Abstracts files for the period 1970 to the present time, as well as a chemical name file, Biological Abstracts and Environmental News, Pharmaceutical News, and Pollution Abstracts.

Results: I was consulted by 64 investigators this year, over twice the number that consulted me last year. Investigators with whom I have collaborated refer others; I consider that recognition for my services by the NIH community. The library refers 'difficult' chemical and pharmacological searches to me as well as some unusual ones. I collaborated with one person to sort out information on one positional isomer out of 16. Another referee needed to know whether a particular diiodothyronine had ever been reported to occur naturally. I did an exhaustive search which indicated that it was not a known natural product.

I have appended a partial list of the topics of interest (more than 150) to our investigators to give an idea of the scope of these investigations. A casual survey of these topics, which I outline below, look routine; many of them are far from that. I refer all monthly updates to the library. I have not counted in this report many quick searches, involving author verification, etc.

At least three review articles were written with considerable aid from me. One author had a two week period to review familial diseases for a chapter in a book. He told me that he never could have completed the review without my prompt assistance in providing a complete, pertinent bibliography. Another author thanked me for my thoroughness in obtaining all of the available literature. A review of asymmetric structural features of the cell membrane was challenging. A reviewer of cyclic nucleotide metabolism asked for my help several times. A bibliography for a kallikrein-kinin review was developed by me. Toxic symptoms which occur upon expansion of extravascular space with albumin were unusually difficult to ascertain. Another referee wanted to find out how the quality of a nursing education could be evaluated which turned out to be a difficult search problem.

My aim is to work with an investigator, determine exactly what information is needed and to obtain pertinent information without a lot of stray material. I try to answer questions precisely, not get large bibliographies.

Partial list of topics searched within the last year:

1. Cyclic nucleotides
2. Structure and availability of epidermal growth factor
3. Phosphorylation of nuclear acidic and non-histone proteins
4. Does suckling release prolactin?
5. Binding of drugs to lung tissue
6. Analysis of data on binding with Scatchard plots
7. Assay of proteins at picogram levels
8. Membranes
9. Angiotensin
10. SRS-A in rabbit aorta
11. Kallikrein and kinins
12. Hypertension
13. Blood cells and hemoglobin
14. Tissue culture
15. Prostaglandins
16. Physical and organic chemistry
17. Reviews of several hereditary diseases
18. Treatment of Anorexia nervosa
19. Therapy to loosen catracts for surgery
20. Are drugs which block collagenase useful to treat arthritis?
21. Is there any correlation between homocystinuria and lung diseases?
22. Treatment of neuroblastoma
23. Toxicologic problems
24. Amines

Significance to Biomedical Research and Institute Program: The increased emphasis on medical research in the last thirty years has generated a large mass of biomedical information. More efficient methods have been developed to determine the current status of a problem. This project speeds up access to the medical and chemical literature. It saves the time of expensive personnel by more rapidly ascertaining whether a project has been done and by examining methods to obtain the most efficient way to answer a question.

Proposed Course of Project: It is proposed to increase the speed of access to computer files. Since the demand for services has more than doubled again this year over last year, I suggest that a cathode ray tube terminal with a larger 'scroll-back' memory and another high speed printer be obtained, both of which operate at 120 cps or higher. Our current equipment is utilized to over 70% of the time so that delays are frequently encountered in getting access to a terminal. The necessity for using automated information data bases, for using computer stored data, and for using computer programs for computation increases yearly in clinical and toxocological research.

Publications:

1. Maling, H.M., Saul, W., Williams, M.A., Brown, E.A.B., and Gillette, J.R. On the mechanism of the potentiation of beta adrenergic angonists of

paraquat toxicity in rats and mice. In Autor, Ann(Ed.): Biochemical Mechanisms of Paraquat Toxicity. New York, Academic Press, 1977, in press.

2. Maling, H.M., Saul, W., Williams, M.A, Brown, E.A.B., and Gillette, J.R. Reduced body clearance as the major mechanism of the potentiation by beta-2-adrenergic agonists of paraquat lethality in rats. Toxicol. Appl. Pharmacol., 1977, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01911-01 HE

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Studies of Kallikrein and Kinins in Hypertensive and Normotensive Humans

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

PI:	David Horwitz, M.D.	Senior Investigator	HE NHLBI
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	Joseph Vinci, M.D.	Staff Associate	HE NHLBI
	Randall Zusman, M.D.	Staff Associate	HE NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Hypertension-Endocrine Branch

SI

Section on Experimental Therapeutics

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	.75	.75

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The vasodilator kallikrein-kinin system was studied in white and black hypertensive and normotensive subjects. Excretion of urinary kallikrein was significantly lower in black than in white subjects (5.4 vs 10 TU/day, $p < .01$) but did not correlate with excretion of kinins and neither correlated with blood pressure. Black hypertensives had values of plasma bradykinin twice those of normal subjects or white hypertensives ($p < .001$). Urinary kallikrein doubled during restriction of dietary salt but urinary kinins and plasma bradykinin remained unchanged. The findings are consistent with racial differences in the kallikrein-kinin system but do not support decreased vasodilator activity of the system as a factor predisposing to hypertension.

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Objectives: Kallikreins are enzymes that produce the potent vasodilator kinins, bradykinin and lysyl-bradykinin. Kinins have been considered to have a possible role in the function of the kidneys and in regulation of blood pressure. Urinary kallikrein is formed by the kidneys, differs from plasma kallikrein, and has been reported to be reduced in patients with essential hypertension. Increases in excretion of urinary kallikrein occur during salt deprivation and are thought to be mediated by increases in levels of aldosterone. Acute changes in levels of plasma bradykinin have been found after infusions of salt to sodium depleted subjects and after postural change but effects on urinary kinins have not been reported. The present study was intended to compare the responses of the kallikrein-kinin system in hypertensive and normotensive subjects and to study the relationship of levels of urinary kinins to spontaneous and induced levels of urinary kallikrein.

Methods: Eighteen patients (nine black, nine white) with essential hypertension were matched by age, sex, and race with eighteen normal subjects. Subjects were studied after at least three days of diet containing 109 mEq of sodium and 100 mEq of potassium and again after five days of daily intake of 9 mEq of sodium and 100 mEq of potassium. During two subsequent days of each period urine was collected for assay for kallikrein, kinins, aldosterone, sodium, potassium, and creatinine. On the last day of each period blood samples were drawn after subjects had been recumbent overnight and again after they had been upright for three hours; blood samples were assayed for plasma renin activity, aldosterone, and plasma bradykinin. Urinary kallikrein was assayed by a modification of the esterolytic assay of Beaven et. al. whereas plasma renin activity, urinary and plasma aldosterone, urinary and plasma kinins were measured by radioimmunoassay. The assays of renin and aldosterone were performed by Hazelton Laboratories, Inc. under a special contract.

Major Findings: 1) Urinary kallikrein and kinin - Excretion of urinary kallikrein was similar in hypertensive and normotensive subjects but was significantly lower in black than in white subjects (5.4 vs. 10.0 TU/day; $p < .01$). Excretion of urinary kinins was similar overall when hypertensive and normotensive and when black and white subjects were compared, but was moderately lower in black than in white hypertensives (11.5 vs. 15.8 µg/day, $p < .05$). Excretion of aldosterone was similar in the black and white subjects (12.3 vs. 12.1 µg/day, n=24).

Urinary Kallikrein Excretion (109 mEq Na).

TaME Units/Day

	BLACK	WHITE	TOTAL
Hypertensive	5.6 ± 1.1 (n=9)	10.9 ± 2.1 (n=8)	8.1 ± 1.3 (n=17)
Normotensive	5.2 ± 0.7 (n=8)	9.1 ± 1.0 (n=9)	7.3 ± 0.8 (n=17)
Total	5.4 ± 0.7 (n=17)	10.1 ± 1.1 (n=17)	

Course of Project: 1) Characterization of the kallikrein-kinin system in more patients with different types of hypertension with special attention to low vs. normal renin hypertensives.

2) Assessment of the amount of urokininogen and the activity of urinary kininases in the urine of patients and normals to determine the factors that control urinary kinin excretion.

Publications:

1. Gillin, J.C., Horwitz, D., and Wyatt, R.J.: Pharmacologic Studies of Narcolepsy Involving Serotonin, Acetylcholine, and Monoamine Oxidase. In Guilleminault, C., Dement, W.C., and Passouant, P. (Ed) Narcolepsy. Advances in Sleep Research, Vol 3, 1976. Spectrum Publications, Inc., New York.

2. Horwitz, D., Thorgerirsson, S.S., and Mitchell, J.R.: The Influence of Allopurinol and Size of Dose on the Metabolism of Phenybutazone in Patients with Gout. Eur. J. Clin. Pharmacology. In press, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01912-01 HE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Involvement of Prostaglandins in the Vasodilator Action of Some Anti-hypertensive Drugs

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

PI:	Barbara Slack, Ph.D.	Visiting Fellow	HE NHLBI
OTHER:	Harry R. Keiser, M.D.	Deputy Chief	HE NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine Branch

SI
Section on Experimental Therapeutics

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.50	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)

Studies were performed on rats and dogs to determine if prostaglandins mediate the vasodilator action of certain anti-hypertensive drugs. The prostaglandin synthetase inhibitor, indomethacin, had no effect on the anti-hypertensive action of hydralazine in spontaneously hypertensive rats but it reduced the anti-hypertensive action of hydralazine in our spontaneously hypertensive dogs. This suggests that prostaglandins may mediate some of the anti-hypertensive action of hydralazine in certain species of animals.

Objectives: It is well documented that the major antihypertensive action of hydralazine is via direct relaxation of vascular smooth muscle, the greatest effect being on the arterioles. This vasodilator action is still apparent after vasomotor control has been chronically impared by sympathectomy or by spinal cord section. It seemed possible that this vasodilatation could be mediated by local production of prostaglandins in the arterioles.

The following studies were designed to determine whether the antihypertensive action of hydralazine could be blocked by administration of the prostaglandin synthetase inhibitor indomethacin.

Methods: 1) Acute studies in spontaneously hypertensive rats - Paired studies were carried out on 6 female spontaneously hypertensive rats. Three separate studies were conducted in each rat to determine a) the response to hydralazine (0.2 mg.IP) alone; b) the response to indomethacin (0.5 mg.IP alone; c) the response to a combination of hydralazine and indomethacin.

For this purpose, the rats were warmed to 37°C for 5 minutes and the basal systolic blood pressure and heart rate were measured by indirect tail plethysmography before any drugs were given and at 20, 40 and 60 minutes after therapy.

2) Chronic studies in spontaneously hypertensive rats - Five spontaneously hypertensive rats were given 0.5 mg indomethacin intraperitoneally daily for 4 days. On day 4, after administration of indomethacin, the rats were warmed as in 1 and basal systolic blood pressure and heart rate measured. 0.2 hydralazine was then injected intraperitoneally. After 30 minutes, blood pressure and heart rate were measured.

3) Control study to ensure blocking of the prostaglandins. Six spontaneously hypertensive rats were divided into two groups of rats. To one group 0.5 mg of indomethacin was injected i.p., the second group received an injection of vehicle (ethanol and prosphate buffer) ip. After 30 minutes, 2 ml blood was withdrawn by cardiac punction from each rat for prostaglandin estimation.

4) Acute Studies in Dogs - Six female fox hounds were randomly selected with mean arterial blood pressures (MAP) (pentobarbitone anaesthesia) above 130 mm Hg. Jugular and vein femoral artery catheters were implanted, and the dogs allowed to reach a steady resting state. Basal MAP and heart rate were measured for 10 minutes and 10 ml of blood withdrawn for prostaglandin estimation then 50 mg indomethacin injected intravenously. After 30 minutes a further blood sample was taken for PG. After 50 mins. 20 mg hydralazine was injected intravenously and at +80 minutes blood taken for PG estimation. MAP and heart rate were monitored continously, and the experiment ended at +120 minutes.

The experiment was repeated for each dog, on a separate occasion, substituting a control vehicle solution for the indomethacin.

Major Findings:

Acute and chronic studies in rats - The mean percentage fall in systolic blood pressure in 6 rats 20 minutes after injection of hydralazine alone was -22.3 ± 6.7%, 20 minutes after indomethacin alone was 2.6 ± 4.9 and 20 minutes after indomethacin and hydralazine 28.9 ± 4.7. Thus indomethacin had no significant effect on the hypotensive action of hydralazine. After chronic indomethacin for 4 days the mean % fall in systolic blood pressure was -29.7 ± 4.6%.

Acute studies in dogs - In two out of six dogs the blood pressure was refractory to hydralazine. In the remaining 4, the mean % fall in mean arterial pressure in response to hydralazine after pre-treatment with indomethacin was -10.1 ± 5.3% and the mean % increase in heart rate was 44 ± 11.9%. The mean % fall in MAP in response to hydralazine without indomethacin was -17.4 ± 3.4% and the mean increase in heart rate 26.5 ± 10.1%.

Prostaglandin measurements - Results awaited.

Significance of Findings to Biomedical Research: In the rat, neither acute or chronic inhibition of the prostaglandins appeared to alter the anti-hypertensive effect of hydralazine. In the dog, however, acute inhibition of the prostaglandins appeared to attenuate the response to hydralazine. This attenuation of blood lowering effect occurred in the absence of any attenuation of the effect on heart rate. This study suggests that there is a species difference in the mode of action of hydralazine and that in the dog, part of its antihypertensive effect is mediated by the prostaglandins. It also seems possible that hydralazine has some direct effect on heart rate not related to its vasodilatory effect.

Proposed Course: It is now proposed to repeat this study using different antihypertensive agents, firstly diazoxide, and then prososin and miaoxidil.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01913-01 HE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Study to Determine if Angiotensin I acts as a Competitive Inhibitor of Converting Enzyme (Kininase II) in vivo.

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

PI:	Barbara Slack, Ph.D.	Visiting Fellow	HE NHLBI
OTHER:	Harry R. Keiser, M.D.	Deputy Chief	HE NHLBI
	Joseph Vinci, M.D.	Staff Associate	HE NHLBI

COOPERATING UNITS (if any)

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Hypertension-Endocrine Branch

SE
Section on Experimental Therapeutics

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.65	0.40	0.25

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Studies were performed in sheep to determine if an infusion of angiotensin I would competitively inhibit angiotensin converting enzyme (kininase II) and produce an elevation in plasma bradykinin. The angiotensin I infusion produced an elevation in blood pressure and in angiotensin II levels but no change in plasma bradykinin or in the breakdown of I^{125} bradykinin.

PHS-6040
(Rev. 10-76)

Objectives: The conversion of angiotensin I to the active component angiotensin II is mediated by converting enzyme. This same enzyme also called kininase II cleaves bradykinin into inactive fragments. In vitro angiotensin I has been shown to be as powerful a competitive inhibitor of converting enzyme as the commercially available SQ 20881 converting enzyme inhibitor. If this were also true in vivo, it could explain why in many experimental and pathological states an increase in plasma angiotensin I and II is accompanied by an increase in plasma bradykinin.

This study was designed to investigate the effect of angiotensin I on the breakdown of bradykinin across the lung.

Methods: Six young sheep in a weight range 13-19 kg were used. These were maintained under pentobarbitone anaesthesia and were implanted with right atrial catheters via the external jugular vein, aortic arch catheters via the carotid artery, femoral artery, and vein catheters. Control blood samples were taken from the aortic arch catheter for angiotensin, prostaglandin E, aldosterone, converting enzyme and continuous blood pressure monitoring started from the femoral artery. 70 µci I^{125} bradykinin was then injected into the right atrium in a bolus sample, in one-seond fractions were immediately taken from the aortic arch into 9 ml of a 75% solution of acetone. Sampling was continued up to 16 seconds after I^{125} bradykinin injection. A 30 ng kg^{-1} min^{-1} infusion of angiotensin I via the femoral vein was then started. After 20 minutes a series of samples for residual I^{125} bradykinin, angiotensin II, prostaglandin E, aldosterone and converting enzyme were taken. A second 70 µci I^{125} bradykinin injection was given and samples collected as before. Samples taken after both I^{125} bradykinin injections were processed as follows:

Samples were centrifuged at 10,000 rpm for 10 minutes at 4° C The supernantant was removed and a fraction counted for total counts in a liquid scintillation counter 5 ml of the supernatant was flash evaporated in a siliconised glass tube. This was then reconstituted with 500 µl of a 0.05 M ammonium acetate solution pH5. This was run over a 2 cm CM sephadex column previously equilibrated with 0.05 M ammonium acetate pH5 buffer. Bradykinin fragments (carrying the I^{125} molecule) were eluted with 6 ml of 0.2M ammonium acetate pH5. Intact bradykinin was eluted with 4 ml 0.5M ammonium acetate pH 7.2. Both fractions were counted and expressed as a percentage of the total counts to give % fragmentation compared to total injected I^{125} bradykinin.

Major Findings: Control angiotensin II, aldosterone, prostaglandin E, converting enzyme; results awaited.

During angiotensin I infusion blood pressure increased by an average of 20 mm Hg from control levels. Angiotensin I levels measured in a pilot study, showed that at an infusion rate of 30 ng Kg^{-1} min^{-1}, angiotensin II levels increased from a control value of 57 pg/ml to >250 pg/ml. In the control state, fragmentation of bradykinin as measured by the above technique was 64 ± 8 (mean, SEM). After a 20 minute angiotensin infusion fragmentation was 62 ± 4%.

Significance of Findings: In vivo it appears that angiotensin I does not act as a competitive inhibitor for a converting enzyme thereby decreasing plasma bradykinin breakdown, and increasing plasma bradykinin levels. This is in contrast to in vitro studies where angiotensin I has been shown to be a powerful competive inhibitor. Thus an alternative mechanism must be postulated for the experimental observation of an increase plasma bradykinin in association with increased plasma angiotensin II levels.

Proposed Course of Project: To investigate the discrepancy between the in vivo and in vitro observations.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01914-01 HE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
The Development of a Radioimmunoassay for Angiotensin II and its Measurement in Normal Subjects and Subjects on Fixed Sodium Intakes

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

PI:	Barbara Slack, Ph.D.	Visiting Fellow	HE NHLBI
OTHER:	Harry R. Keiser, M.D.	Deputy Chief	HE NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine Branch

SI
Section on Experimental Therapeutics

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.50	0.25	0.25

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A radio-immunoassay for angiotensin II has been established in our laboratory. The characteristics of the antibody and the procedures for collection of samples and extraction of the angiotensin II for assay have been fully investigated and optimized. Values for normal subjects on various sodium intakes have been determined and they correlate well with those reported by others.

Objectives: Angiotensin II is the most potent pressor substance known. It is 80x more potent than norepinephrine on a molar basis. It is also a major stimulant of aldosterone and interacts with other hormones in the kidney to modify sodium excretion. In research concerned with the causes of hypertension, any hormone potentially capable of raising blood pressure and altering sodium handling in the kidney is of primary importance, and it was considered useful to set up a sensitive assay for measuring plasma levels in man and other animals.

Methods: Collection of Blood Samples - Venous or arterial blood samples were collected into a solution containing 0.125 M EDTA (disodium salt) and 0.05 M 1-10 phenanthroline. This solution has been shown to effectively inhibit converting enzyme and angiotensinases in vitro. The solution was used with blood in a ratio 1:20. Blood samples were centrifuged at 2000 rpm at 4° C as soon as possible, and the supernatant plasma decanted and froze.

Extraction of Angiotensin II - Measured amounts of plasma were extracted in a batch extraction on 400-450 mg Dowex Ag50 resin (50-100 mesh). Approximately 2000 cpm I^{125} angiotensin II was added as a recovery marker. Angiotensin II was subsequently eluted from the Dowex with 2ml of a 10% methanolic ammonia solution, and the eluate dried under a stream of nitrogen.

Assay System - The extracts were reconstituted with 1 ml of Dulbecco's PBS with added calcium and magnesium. 500 µl aliquots were incubated at 4° C with 100 µl rabbit anti-angiotensin II antiserum (final dilution 1:900,000) and 400 µl I^{125} angiotensin II (approximately 10 pg I^{125} angiotensin II) (New England Nuclear).

The resultant binding of unknown angiotensin to antiserum was compared with a standard curve constructed using standard angiotensin in a range 3.1 to 200 pg/tube. Free angiotensin II was separated from angiotensin II bound to antiserum using a dextran charcoal mixture. Free I^{125} angiotensin II in the charcoal was counted using a gamma scintillation counter.

Experiments: 1) 2 x 20 ml of blood was removed from 10 normal standing laboratory workers on no fixed sodium intake and placed into inhibition solution. The plasma was separated, extracted, and assayed for angiotensin II levels.

2) 2 x 20 ml of blood was removed from 5 normal supine subjects on a diet containing 109 mEq sodium/day and from 7 normal supine subjects on a diet containing 9 mEq sodium/day. The samples were processed as above and assayed for angiotensin II.

Major Findings: The assay system yielded a good sensitive standard curve for angiotensin II in the range 3.1 to 200 pg; the curve starting at 50% binding for 0pg angiotensin and ending at 5% binding for 200 pg.

Angiotensin II levels Normal Standing Subjects, no fixed sodium intake- Plasma angiotensin II on 20 samples from 10 subjects gave a mean level of 17.7 ± 2.6 (SEM) pg/ml with a mean recovery of I^{125} angiotensin II from plasma of 84.1 ± 3.1%. The range was between 1.0 and 37.7 pg/ml.

Angiotensin II Levels Normal Supine Subjects, dietary sodium 109 mEq/day - Plasma angiotensin II on 9 samples from 5 subjects gave a mean level of 27.8 ± 2.9 pg/ml with a mean recovery of 82.8 ± 2.2%. Range = 19.0 - 34.4 pg/ml.

Angiotensin Levels Normal Suping Subjects, dietary sodium 9 mEq/day - Plasma angiotensin II on 14 samples from 7 suhjects gave a mean level of 99.3 ± 21.8 pg/ml with a mean recovery of 82.8 ± 2.1%. Range = 31.4 - 267 pg/ml.

Significance of Findings: This assay provides a sensitive method of assaying angiotensin II in plasma capable of detecting levels from pg/ml to > 250 pg/ml. Recoveries of added I^{125} angiotensin II are high and consistent. As recorded previously, angiotensin II levels are directly related to sodium intake with higher levels being recorded in states of sodium depletion. As shown by the larger standard error on values from sodium depleted subjects, there is a degree of individual variation in the response of angiotensin II to sodium depletion.

Proposed Course of Project: Work is continuing to improve the accuracy of this assay and simplify the extraction technique. A similar assay system is being developed for the precursor of angiotensin II, angiotensin I.

Both assays will subsequently be used for the measurement of angiotensins in various pathological and research conditions, and for correlations with other renal hormones involved in blood pressure homeostasis.

Publications: None

ANNUAL REPORT
SECTION ON BIOCHEMICAL PHARMACOLOGY
HYPERTENSION-ENDOCRINE BRANCH
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

The primary objectives of this section's research program are:

1) To determine the molecular mechanisms that regulate both the activity and amount of enzymes involved in the biosynthesis of aminergic neurotransmitters in various cell types and tissues.

2) To understand the molecular events responsible for the cellular effects of aminergic neurotransmitters on target cells and organs.

3) To investigate the role of both central and peripheral aminergic neuronal systems in the regulation of blood pressure, and the potential pathogenic role these systems play in the development of hypertension.

4) To develop techniques to measure both central and peripheral activity of the aminergic systems in man. It is hoped that the latter approach will allow us to evaluate the role of the aminergic systems in essential hypertension and other disease states.

These four rather broad objectives have formed the basis for a number of integrated projects. The progress toward the achievement of these objectives will be presented sequentially although it should be noted that many of the projects relate to more than one of the objectives.

I Molecular Properties of Hydroxylases Involved in Neurotransmitter Synthesis

The work of our section is primarily directed toward the neurotransmitters dopamine, norepinephrine, epinephrine and serotonin. The enzymes of primary interest in the synthesis of these neurotransmitters are tyrosine hydroxylase, dopamine-β-hydroxylase, phenylethanolamine-N-methyl transferase, and tryptophan hydroxylase. A characterization of the molecular properties of these four enzymes is one of the continuing goals of this section.

Tyrosine hydroxylase is the first and rate limiting enzyme for the synthesis of all catecholamines. It has been reported that the molecular properties of this enzyme are different in various brain regions and the adrenal gland. The sedimentation coefficient and Stoke radius of tyrosine hydroxylase from brain and adrenal gland were determined by sucrose gradient centrifugation and gel filtration respectively. Tyrosine hydroxylase has a molecular weight of approximately 216,000 daltons. The molecular parameters of the enzyme were similar when the source was either the adrenal gland or any brain region. Since tyrosine hydroxylase appears to have subunits of about 60,000 daltons, our tentative conclusion is that the active enzyme is a tetrameric molecule. In a separate line of experimentation we examined the properties of tyrosine hydroxylase from cultured neuroblastoma cells. In contrast to the enzyme from brain or adrenal this enzyme appeared to be entirely associated with the particulate fraction and extremely heat labile in the broken cell extracts. However the enzyme appeared to mechanistically similar to that of the brain and exhibited the normal end-product inhibition.

Dopamine-ß-hydroxylase, which catalyzes the final step of norepinephrine synthesis is easily isolated as a pure protein and has been characterized previously by our section as a protein consisting of four apparently identical subunits with significant carbohydrate side chains. In the past year we have completed the analysis of the molecular parameters of the enzyme in human serum. Two molecular weight species exist in human serum, one having a molecular weight of 298,000 (80%) and another with a molecular weight of 147,000 (20%). The Stokes radii and sedimentation coefficients for these two species are: 65.1 Å and S_{20w}=10.3, and 50.1 Å and S_{20w}=6.8. For comparative purposes the pure enzyme isolated from bovine adrenal glands has a Stokes radius of 61.3 Å and a sedimentation coefficient (S_{20w}) of 10.1 and a calculated molecular weight of 267,000. From these studies and earlier ones in which we observed that the monomeric unit of human dopamine-ß-hydroxylase had a molecular weight of about 75,000 we conclude that human dopamine-ß-hydroxylase exists in serum as dimers and tetramers both of which have catalytic activity. Our studies also suggest that since the currently accepted mechanism involves the participation of two copper atoms and since there appears to be one copper atom per subunit, that two subunits must cooperate to form an active site.

The interaction of substrate with the copper of the active site of DBH has been investigated by electron paramagnetic resonance (EPR) spectrometry. Deoxygenation of freshly prepared enzyme causes a decrease in the magnitude of the enzymic cupric signal. Addition of the substrate, tyramine enhances the magnitude of the EPR signal with the appearance of super hyperfine lines. This phenomenon is consistent with the interaction of the substrate nitrogen with the enzymic copper. Clearly the interaction of the substrates oxygen and tyramine with enzyme occur in close proximity to the copper binding site.

Tryptophan hydroxylase from brain has also been resistent to total isolation. Chromatography of semipurified enzyme on Sepharose-4B indicated that this enzyme has a molecular weight of 240,000-270,000 daltons. Of interest is the observation that the substrate tryptophan appears to dissociate this hydroxylase into smaller subunits.

II Kinetic and Regulatory Properties of Tyrosine and Tryptophan Hydroxylase

The activity of tyrosine hydroxylase within neuronal cells is severely curtailed by the concentration of free catecholamines and the hydroxylase cofactor, tetrahydrobiopterin. These factors are related since the end-products (catacholamines) compete with the cofactor for a binding site on the enzyme. Our laboratory had previously found that the apparent affinity of tyrosine hydroxylase for its cofactor was controlled by a c-AMP dependent protein phosphorylation. During the past year we have examined this phenomenon in greater detail and found that protein phosphorylation had little effect on the K_m for 6-methyltetrahydropterin when the enzyme was first separated from endogenous catacholamines. Kinetic analysis of semipurified or Sephadex G-25 treated enzyme indicated that protein phosphorylation significantly increases the end-product K_i value. These findings provide a molecular basis for the previously observed phenomenon that following certain physiologic stimuli, the in vivo synthesis of catecholamines becomes less sensitive to end-product concentration.

In vivo the regulation of tyrosine hydroxylase is mediated by neurotransmitter receptor activation or blackade. An example of this is the well known

affect of dopamine receptor blockade on the kinetic properties of tyrosine hydroxylase in dopaminergic nerve terminals. Our experiments are consistent with, but do not prove that such regulatory mechanisms are mediated by protein phosphorylation. In rats haloperidol causes a dramatic increase in the apparent affinity of tyrosine hydroxylase for tetrahydrobiopterin in the caudate nucleus. This increase is apparent 1 hour following drug treatment. However by 24 hours the kinetic state of the enzyme has returned to normal. We have found that 1 hour after the last of 10 daily doses of haloperidol there are increases both in the amount of enzyme and in its cofactor affinity. Conversely 24 hours after the last of ten daily doses, the amount of enzyme remains elevated, but K_m has risen to four times the control value. It is interesting that haloperidol is largely ineffective in psychotic patients for the first several days of treatment suggesting that its therapeutic efficacy may be related to a biochemical adaptation. Treatment of rats with LSD resulted in an activation of the kinetic properties of tyrosine hydroxylase. This is consistent with results in other laboratories showing that LSD treatment increased dopamine turnover, and with the concept of an interaction of the dopamine and serotonin systems. It is now clear that there are many regulatory systems that impinge upon dopamine neurons and control the rate of neurotransmitter synthesis by changing the kinetic properties of tyrosine hydroxylase. We have gathered circumstantial evidence that these control systems may function by means of phosphorylation of tyrosine hydroxylase.

Considerably less is known about the molecular mechanism of regulation of tryptophan hydroxylase. We have recently obtained evidence that this enzyme is also actively regulated. Protein phosphorylating conditions can cause kinetic activation of the enzyme. In this case there appears to be little significant end-product inhibition, and changes in the affinity for cofactor have a direct effect on reaction rates. A somewhat similar activation of tryptophan hydroxylase is observed by the addition of Ca^{++} and it has been suggested that this activation may be due to a proteolytic reaction.

III Effects of Transmitters on Target Cells

Induction of pineal serotonin-N-acetyl transferase (SNAT). In the pineal gland the synthesis of the hormone melatonin is controlled by sympathetic innervation. Activation of β-receptors on the gland results in an increase in intracellular cAMP concentration, and a delayed increase in SNAT. We have used an organ culture system to define the molecular mechanisms which function between the release of cAMP and the new enzyme synthesis. As a first step, we determined the size of pineal SNAT. Because of a very short half-life of the enzyme in solution a miniaturized rapid gel filtration system was developed. The molecular weight of rat pineal SNAT is about 39,000 daltons. The enzyme however can be dissociated into a subunit with a molecular weight of 10,000. In contrast N-acetyl transferase from liver appears to be a dimer with subunit mulecular weight of 13,000.

Addition of isoproterenol to the culture system results in a 50 fold increase in SNAT activity. A careful electrophoretic analysis of the newly synthesized protein indicated that no one protein was preferentially synthesized. There was a small but significant increase in overall amino acid incorporation. Specific examination of newly synthesized polypeptides with molecular

weight in the range of 10,000 also did not yield any suggestion that a specific protein was being synthesized.

The incorporation of uridine into the various species of RNA was examined with no preferential synthesis being observed during the induction phenomenon. Concentration curves with various specific inhibitors of RNA synthesis revealed that at certain concentrations of actinomysin D, α-amanitin, and cordycepin, 70-85% inhibition of messenger RNA synthesis could be obtained with little or no effect on the induction phenomenon. These experiments together with the protein synthesis work suggest that the "apparent induction" of SNAT may not involve the synthesis of new protein or RNA molecules. Another line of experiments however were consistent with a nuclear event being responsible for the increase in SNAT activity. In these experiments $^{33}PO_4$ was included in the pineal organ culture during the induction process and the distribution of labelled proteins examined. It was shown that a specific nuclear protein was phosphorylated prior to the apparent increased enzyme activity. This nuclear protein has a molecular weight of about 34,000 daltons.

Our current hypothesis of how nerve impulses are translated into changes in SNAT activity in the pineal gland is: 1) activation of β-receptors: 2) stimulation of cAMP synthesis; 3) activation of a protein kinase; 4) phosphorylation of a chromatin protein; 5) increased synthesis of SNAT messenger RNA and increased SNAT. Our experiments with pineal protein and RNA synthesis do not support the above hypothesis while our experiments on nuclear protein phosphorylation are consistent with the hypothesis.

Regulation of tyrosine hydroxylase content in the rat carotid body. The carotid body serves as a oxygen sensor in mammals. Previously we had reported that dopamine was the major biogenic amine in this organ and that exposure of rat to hypoxic conditions results in long-term increase in tyrosine hydroxylase activity in this organ. This increase in activity is dependent upon new protein synthesis. Hypoxia appears to cause a release of dopamine. While it was impossible to measure cAMP changes directly we found that shortly after exposure to hypoxic conditions, the phosphodiesterase of the carotid body was activated. This is indicative of a transient increase in protein kinase activity reflecting synthesis of cAMP. A subsequent event in the induction process appears to be a general increase in RNA polymerase II dependent RNA synthesis. The induction of tyrosine hydroxylase, the increased RNA synthesis and the activation of phosphodiesterase are all dependent upon intact innervation of the carotid body. It therefore appears that the carotid body adapts to its need for increased dopamine synthesis by a process which is similar to the induction of SNAT in the pineal gland. This work also begins to provide a molecular explanation for mechanism by mammalian organisms adapt to prevailing oxygen concentrations.

IV Neuronal Regulation of Blood Pressure in Experimental Animals

In previous years we have used the spontaneously hypertensive rats (SHR) as models for certain parameters of human essential hypertension. Transient increases in tyrosine-hydroxylase and DBH have been observed in the hypothalamus during the developmental phase of hypertension in these animals. There is a highly significant correlation between increment in blood pressure from 3 to

8 weeks of age and the increase above control level of the hypothalamic content of these two enzymes. By twelve weeks of age when the animals are severely hypertensive the content of these two enzymes is similar to that in control animals in all brain regions. We speculate that these early changes in the brain neurotransmitter systems may be related to the pathogenesis of hypertension. It is likely that any change in the central nervous system that affect blood pressure is expressed via changes in sympathetic outflow to the peripheral vasculature. Increase sympathetic outflow in turn may cause either an acute pressor effect or long-term changes in the structure of the vasculature. Previous studies in our laboratory and others suggest at least transient increases in sympathetic nerve activity during the developmental phase of hypertension in the SHR. We have been particularly interested in the neuronal effects on vascular protein synthesis and in previous years had shown that the SHR had a significantly higher rate of lysine incorporation into the non-collagen protein, in the mesenteric vessels. During the past year we have found that the intensely innervated testicular arteries in the SHR show an approximately 3 fold greater rate of incorporation of lysine into non-collagen protein than do arteries from Wistar-Kyoto rats. The effect of several antihypertensive agents on the increased rate of non-collagen protein formation in the testicular artery was examined. Clonidine, a drug which acts by stimulating the α-receptors in the brain and thereby inhibiting sympathetic outflow was effective both in normalizing the blood pressure and the rate of lysine incorporation into vascular non-collagen protein. Similar studies with the ganglionic blocking agent hexamethonium indicated that this agent also was effective in slowing non-collagen synthesis. Conversely hydralazine, a vasodilator, was a very effective antihypertensive agent, but had no effect on amino acid incorporation. These studies were consistent with the concept that sympathetic input to small vessels had an effect on the protein metabolism and thereby on the structure of the small resistance vessels. The effect of either α- or β-receptor blockers was next examined. In this study phenoxybenzamine was found to be effective in both reducing blood pressure and the rate of lysine incorporation into non-collagen protein in the testicular artery. Propranolol however was ineffective in reducing blood pressure and had no effect on lysine incorporation. It is interesting to note that histological studies indicated a good correlation between vascular thickening and lesions with the rate of lysine incorporation measured under the various experimental conditions. From these studies a picture is beginning to emerge suggesting that the development of hypertension in this animal model occurs as a result of an abnormality in the central nervous system leading to change in sympathetic activity and that the primary effect of the sympathetic outflow is an increase in the formation of structural or contractile components of the small resistance vessels. The molecular mechanism by which these changes occur remain to be elucidated and there is no certainty that this phenomenon even represents one of the contributing factors to human essential hypertension.

V Measurement of Indices of Aminergic Activity in Man

Measurement of neurotransmitters and their metabolites in urine, blood or cerebrospinal fluid has been the classic approach to an evaluation of the activity of neurotransmitter systems in man. Data obtained from blood or urine are somewhat ambiguous since they represent both central and peripheral activity. Cerebrospinal fluid while providing a more direct measure of central activity has not been widely used.

We have attempted to use serum-norepinephrine and -DBH as indices of peripheral sympathetic activity. Norepinephrine appears to be a good index of short-term sympathetic activity. DBH cannot be related to short-term activity and may not be a good index of longer-term sympathetic activity. These indices were evaluated in groups of age, sex, and race matched normotensive and hypertensive subjects. No significant correlation of these indices with blood pressure were observed.

In an attempt to assess the noradrenergic activity of the brain we developed an assay system that had sufficient sensitivity to measure DBH in cerebrospinal fluid. The activity of this enzyme has now been measured in the CSF of over 60 human subjects (psychiatric patients and alcoholics). The amount of enzyme in CSF is about $\frac{1}{1000}$ of that found in serum. No significant differences have been observed in any of the diagnositic sub-groups. Manic-depressive subjects, however, appear to have lower CSF-DBH during the manic phase of their illness. This returns toward normal during their recovery. There appear to be no age or sex differences in enzyme content, however there is a slight correlation between DBH activity in serum and CSF. There also may be sub-group of schizophrenic subjects with low CSF-DBH. This would be extremely interesting since one of the current hypotheses concerning this disease is that there is a relative deficiency of this enzyme in certain brain regions. Preliminary data also suggest that treatment of subjects with monoamine oxidase inhibitors results in a decrease in the content of this enzyme in CSF. Such an observation would be consistent with receptor-mediated regulation of the central noradrenergic system. The potential of this system for exploring human essential hypertension and the response of this disease to centrally acting drugs is obvious.

In another approach to the evaluation of central aminergic activity in man we have developed a technique for the measurement of the hydroxylase co-factor, tetrahydrobiopterin, in CSF. Since our biochemical studies have established that this compound plays a key role in regulating neurotransmitter synthesis, it was of particular interest to have a means of evaluating its content in the human central nervous system. For these studies we have used a purified phenylalanine hydroxylase system which allows the measurements of as little as 5 pmoles of cofactor. The CSF from 30 neurologic and psychiatric patients have been examined to date. While the data are too preliminary to draw definitive conclusions, it is interesting to note that individuals with basal ganglia disease have significantly lower CSF-cofactor amounts, this is of course consistent with the well established fact that these individuals have a substantial loss of the central dopaminergic systems.

While our attempts to devise indirect indices of central aminergic activity in man are in their beginning stages, it appears that such approaches will lead to considerably better understanding of role of aminergic systems in brain function and how these systems relate to human disease.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01843-04 HE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Mechanisms of Transport: Uptake and Release of Biogenic Amines in Nerve Endings

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

PI: Donald F. Bogdanski Pharmacologist HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

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NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

We have been studying a model neurosecretory system for the in vitro secretion of norepinephrine in rat heart slices. The slices are incubated in a Na^+ - deficient ($choline^+$) medium and the secretion is Ca^{++} dependent. Methods and general characteristics of the model have been described in previous reports. It has been shown that neurosecretion has some characteristics of outward transport and some characteristics of synaptic transmission. Moreover, the model is a high capacity system that can secrete 70% of the stored NE within 140 min. In view of the fact that serious questions remain for the establishment of exocytosis as the secretory process in nerve endings, outward transport may be considered a potential mechanism for the physiological secretion of transmitter.

In case of malfunction of the storage process, moreover, the norepinephrine in vesicles attached to the synaptic membrane could be transported into the synaptic cleft.

Objectives: To illustrate some similarities in the process of model neurosecretion and synaptic transmission.

Major Findings: Current findings support previous findings on the parallel characteristics of the two processes. Secretion in the model system is stimulated by intracellular Na^+ in accord with the view that secretion is mediated by outward transport. In Na^+ loaded slices the Na^+-dependent component of efflux was inhibited by cocaine, a specific inhibitor of transport. Cocaine, like desipramine had little effect upon the efflux of NE in slices loaded with choline or Li^+. These findings strongly support the concept of carrier mediated neurosecretion. As a corollary, the evidence also indicates the integrity of the plasma membrane exposed to adverse physiological conditions.

Parallels between uptake and secretion

Previous evidence indicates that the uptake inhibitors cocaine and desipramine inhibit uptake and efflux at a similar concentration range.

An additional parallel between uptake and secretion was sought in a known characteristic of efflux. This characteristic tendency for the efflux to slow down as a function of time rather than as a function of amine remaining in the tissue. This tendency is very strong in the presence of Ca^{++}, which also stimulates secretion and can substitute for Ca^{++} in synaptic transmission. To test for parallel inhibition of uptake, slices were preincubated in Krebs-bicarbonate medium then transferred to beakers containing Ca^{++} in a Na^+ -deficient medium. Norepinephrine - ^3H was added immediately or after 100 min. incubation in the Na^+ deficient medium. Pre-incubation was varied so that total incubation time was constant. One hundred minutes of exposure of slices to the Na^+ - deficient medium containing Ca^{++} inhibited transport by 38%. This could explain the slowing of efflux after about 100 min. of incubation.

Effect of transport inhibitors on the metabolism of amines

In control experiments, the proportion of amines to deaminated metabolities of the NE recovered from the medium varies with increasing duration of incubation. For slices incubated in the Kreb-bicarbonate medium, the proportion of amines decreases with time until it becomes about 17% of the total radioactivity released. This proportion is increased by cocaine during pre-incubation but is not changed after continued incubation as the tissue equilibrates with the medium. This finding reflects the inhibition of re-capture and subsequent deamination of amines. For slices incubated in the Na^+-deficient medium, the increment of radioactivity released in excess of the control release was recovered from the medium as non-deaminated amine. Cocaine blocked this release and the proportion of deaminated metabolities correspondingly increased towards control levels.

Similarities between secretion in the model system and synaptic transmission

Reports by other laboratories indicate that synaptic transmission in the cholinergic and adrenergic neurons is inhibited by lanthanum and manganese. These tri and divalent cations, respectively, also inhibit the $Na^+ - Ca^{++}$ stimulated neurosecretory system.

Bretyllium, which blocks adrenergic transmission by preventing the release of neurotransmitter, exerted a weak inhibitory effect upon the model $Na^+ - Ca^{++}$ stimulated neurosecretion, but not upon efflux that occures in Li^+ loaded slices. Bretyllium was only active in inhibiting uptake at concentrations which are more than that required to block synaptic transmission. This finding is not necessarily fatal to the working hypothesis in view of the fact that Ca^{++} inhibits the effects of Bretyllium. As shown previously in this project, large amounts of Ca^{++} accumulate in slices incubated in Na^+ - deficient media. This Ca^{++} might interfere with the more specific actions of Bretyllium, while permitting Bretyllium to block transport.

A series of experiments have shown that certain characteristics of mobilization and release of neurotransmitter in the model neurosecretory system resemble some characteristics of mobilization and release of neurotransmitter during synaptic transmission. Thus, release is Ca^{++} dependent but is inhibited by high concentrations of Ca^{++}. Release is inhibited by magnesium and manganese. Barium can substitute for Ca^{++} but the effect of Ba^{++} is strongly limited by time. The Ca^{++} dependent release is represented by saturation kinetics and inhibited by Na^+. Bretyllium inhibits release or increases release depending upon conditions. Transport inhibitors in general increase overflow but in some experimental preparations decrease overflow.

Mobilization of Bound Amine

During the course of this project, it was found that slices retained their ability to bind, mobilize and secrete amine after prolonged immersion in unphysiological media at 0°. This was the basis for varying the composition of intracellular electrolytes and metabolities of the glycolytic and citric acid cycles.

In the absence of energy producing metabolities, the efflux of stored radioactivity representing injected ^3H-NE was very rapid. Efflux was inhibited by glucose or pyruvate, but not ATP. Moreover, ATP accelerated efflux of radioactivity in Na^+-loaded slices incubated in Krebs-bicarbonate medium, but ATP inhibited efflux of radioactivity in Na^+-loaded slices incubated in the Na^+-deficient (choline$^+$) medium. Thus, it appeared that the model neurosecretory system could be used to study energy-dependent mobilization of amine. However, the efflux of radioactivity in Na^+-loaded slices incubated in Krebs medium was also equally stimulated by ADP and AMP. Thus, the significance of high energy phosphate for mobilization and secretion in this system is in doubt.

The release was independent of Ca^{++}, suggesting that the nucleotides acted further along the chain of reactions between the entrance of Ca^{++} into the cell and the mobilization of bound NE or that the process is non-specific. In this system, cocaine is a weak inhibitor of efflux. Thus, release is not mediated by the transport mechanism indicated.

A possible answer to the question of why inhibitors of transport do not inhibit synaptic transmission

Inhibitors of transport usually increase the release of nuerotransmitter during physiological synaptic transmission. If the model neurosecretory system mimics synaptic transmission, why don't transport inhibitors block synaptic transmission? First it should be pointed out that cocaine increases the effux of NE in slices incubated in the control krebs-bicarbonate solution containing excess K^+. This preparation, rather than the model neurosectory system appears to mimic synaptic transmission. However, it was discovered that excess K^+ and/or depolorization blocks the effect of desipramine in the model neurosecretory system. The explanation proposed for this effect is as follows: Desipramine is a competitive inhibitor of transport. Desipramine and NE, therefore, attach to the same site on the carrier. The affinity of the carrier site is increased by extracellular Na^+. In support of this hypothesis, we have reported that Na^+ lowers the K_m for NE transport. Potassium decreases the affinity between the carrier and substrate, but the effect of Na^+ is stronger. In the presence of Na^+ and K^+, therefore, the inhibitor can compete with NE for the receptor site on the carrier. Desipramine blocks Na^+ - dependent re-uptake of NE, which is then released into the medium at a more rapid rate. In Na^+-deficient media two effects are observed. Sodium dependent re-uptake is inhibited and a blocker of re-uptake can have little additional effect. Secondly, the inhibitor may have a very low affinity for the carrier in the absence of Na^+. Moreover, excess K^+ may produce a conformational change in the carrier which prevents the carrier from binding desipramine. Presumably, the carrier might still transport some NE, a smaller and simpler molecule, despite the conformational change in the carrier. Also there is some Na^+ at the intracellular surface. This control of electrolyte upon the attachment of molecules to the carrier may explain the failure of desipramine to block synaptic transmission, but might still explain the increased overflow of transmitter in the presence of transport inhibition. The attachment of desipramine to the carrier is cyclical. During the passage of the nerve impulse, a transient decrease in the membrane Na^+ concentration which is followed immediately by a transient increase in the K^+ concentration might tend to decrease the population of carriers having attached inhibitor. The carriers could transport amine out of the nerve ending. Between impulse, in an environment of high Na^+ and low K^+ concentrations the carrier could attach desipramine and re-uptake would then be inhibited. Increased overflow would then occur.

Significance to Biomedical Research and Institute Programs

Investigations of the physiological and biochemical characteristics of nerve endings generally are of great interest because synaptic transmission

is crucial to either the initiation, control or maintenance (or all three) of all bodily functions, both physical and mental. Synaptic transmission is the point of attack for many drugs currently used for a wide variety of illnesses ranging from hypertension to mental disease. Fundamental knowledge of the nature of the biochemical processes controlling synaptic transmission is crucial to the understanding of the actions of existing drugs, and the development of drugs.

The findings reported above together with published reports of inhibition of synaptic transmission by desipramine and apparent inhibition of overflow by cocaine, suggest that synaptic transmission includes a component of outward transport of NE, which can be blocked by transport inhibitors.

Proposed Course of Project

This aspect of the studies of transport mediated release of neurotransmitters will be completed along the lines indicated above and then terminated.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01844-03 HE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Receptors Participating in the Induction of Tyrosine Hydroxylase

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

PI: Ingeborg Hanbauer Staff Fellow HE NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Persistent trans-synaptic stimulation or injection of a high dose of carbohydrate active steroids elicit a long-term increase of tyrosine hydroxylase (TH) activity in superior cervical ganglia (SCG). The induction of TH does not depend on an intact retrograde transport system of trophic substances and is not mediated by a second messenger response. Trans-synpatic stimulation elicits an increase in α-amanitin sensitive RNA-synthesis, whereas dexamethasone failed to do so.

Objectives: The mechanisms whereby cholinergic receptor agonists, chemoreceptor stimulation and glucocorticoids induce TH activity is not yet completely understood. There is evidence that in adrenal medulla various stimuli induce TH by an identical mechanism which involves nicotinic receptor stimulation and the participation of a second messenger. In contrast, in SCG and CB, the regulation of TH is determined by various types of receptors, reflecting the complexity of the synaptic organization of these tissues. This project is carried out to characterize the various biochemical steps leading to an increase in TH activity in SCG and CB elicited by various types of stimuli and to decide whether the increase in enzyme activity is triggered by a different or common biochemical mechanism.

Methods: The concentration of cAMP was determined by radioimmunoassay of the succinylated cyclic nucleotide. TH activity was measured by a radiochemical technique with 6-methyltetrahydropterin as cofactor. RNA-synthesis was determined by the incorporation of ^{3}H-uridine into RNA in absence and presence of low concentrations of α-amanitin. Cyclic nucleotide phosphodiesterase (PDE) activity was measured by a radiochemical procedure. Transsection of the postganglionic sympathetic nerve fibers was performed 5 to 7 days prior to the experiments.

Major Findings: The induction of TH in rat carotid body elicited by hypoxia or glucocorticoids is associated with a second messenger response. This was documented by an early increase of cAMP concentration and by a Ca^{2+} dependent decrease in the K_m for cAMP of PDE. In contrast, in SCG both dexamethasone or nicotine increase TH activity without changing the cAMP content or the kinetic forms of PDE. The elevation of TH activity in SCG or CB elicited by dexamethasone does not involve an increase in α-amanitin sensitive RNA-synthesis. Therefore, it can be proposed that the mechanism by which dexamethasone elicits an increase in TH activity operates rather through blockade of TH breakdown than through synthesis of new enzyme molecules. In CB and SCG the induction of TH triggered by an increased trans-synaptic stimulation depends on activation of RNA-polymerase II dependent RNA synthesis.

Proposed Course of Project: In order to understand the molecular mechanisms of TH regulation by trans-synaptic stimulation and glucocorticoids more detailed studies on the activation of protein kinases, RNA-synthesis and phosphorylation of nuclear proteins will be required. Furthermore, studies on the rate of enzyme degradation are proposed to clarify the different mode of TH regulation by dexamethasone.

Publications:

1. Hanbauer, I.: Induction of tyrosine-3-monooxygenase in superior cervical ganglia after axotomy of postganglionic nerves. Neuropharmacology 15: 509-510, 1976.

2. Hanbauer, I.: Hormonal regulation of tyrosine hydroxylase. Symposium on Biochemical and Function of Monoamine Enzymes (Steamboat Springs) Usdin, Earl and Weiner, Norman (Eds.) Marcel Dekker, Inc.,In press (1977)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01846-03 HE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Molecular Biology of Chemoreceptor Regulation
(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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	W. Lovenberg	Chief,Sect. Biochem. Pharmacol.	HE NHLBI
	E. Costa	Chief, Lab. Preclin. Pharmacol.	LPP NIMH

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Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
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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 molecular mechanisms of chemoreceptor regulation in rat carotid body (CB). Exposure to hypoxic conditions (5% O_2) causes an immediate drop in paO_2 to about 35 mm Hg, a decrease in dopamine (DA) content of CB, but fails to change the norepinephrine (NE) concentration in this tissue. These changes are followed by an activation of c-nucleotide phosphodiesterase (PDE) and an increase in RNA polymerase II-dependent RNA-synthesis. After 24 hours tyrosine hydroxylase (TH) activity in CB is increased. The increase in PDE activity, RNA-synthesis and TH activity requires intact innervation of the CB.

Objectives: The carotid body senses physiologic oscillation of arterial pO_2, pCO_2 and pH. It has been suggested that the glomus cells may participate in the chemoreceptive function of the carotid body, either directly or as modulator of the carotid sinus nerve activity. Since DA was shown to be the major neurotransmitter in rat CB it was of interest to study the molecular mechanisms for the regulation of TH, the rate-limiting enzyme in DA synthesis. Special interest is focused on the role of various types of nerves innervating the CB.

Methods: Before, during and after hypoxia blood was drawn from the carotid artery and pO_2, pCO_2 and pH were analyzed immediately in a blood gas analyzer. The concentration of DA, NE, DOPAC and HVA were measured by GC-mass spectrometry. The turnover rate of DA was determined by measuring the rate of decline of DOPAC during the first 8 minutes following injection of pargyline. RNA-synthesis was measured by incorporation of ^{3}H-uridine into RNA in absence or presence of α-amanitin to block RNA polymerase II. A radiochemical procedure was used for measurements of c-nucleotide PDE as described in project.

For innervation studies unilateral transection of the carotid sinus nerve or ganglionectomy was performed five to seven days prior to the experiments.

Major Findings: Exposure to hypoxic conditions lowers paO_2 to about 25% of control, decreases the $paCO_2$ only slightly, but fails to change the arterial pH. When rats are kept in 5% O_2 for 30 minutes DA content in CB decreases about 70%, but the NE concentration fails to change. Sufficient exposure to hypoxia (two-30 minute periods in 5% O_2) elicits an increase in V_{max} of TH 24 and 48 hours thereafter. The K_m of TH for the cofactor, however, does not change 2 hours or 48 hours after hypoxia. The increase in V_{max} depends on new protein synthesis and requires intact innervation of the carotid body. Since a threshold exposure to hypoxia, which is sufficient to increase TH activity in CB fails to change the enzyme activity in other catecholaminergic tissues such as caudate nucleus, superior cervical ganglia, carotid arteries or adrenal medulla, it can be assumed that hypoxia is a specific stimulus involved in the regulation of CB-TH. The mechanism by which this specific stimulation triggers TH induction is not yet resolved. However, our studies brought indirect evidence for a second messenger participation. One hour following exposure to hypoxia the content of PDE-activator protein in CB cytosol fraction increases. This increase is associated with the disappearance of the high K_m-form of PDE and the prevelance of the low K_m-form lasting 2 hours. Furthermore, these changes in the kinetic properties of PDE require the presence of Ca^{2+}.

Our studies brought also evidence for an increase in RNA-synthesis elicited by hypoxia. Since a low concentration of α-amanitin (2.2 μg/ml) is able to block this increase, RNA polymerase II appears to be responsible for eliciting this increased synthesis.

Unilateral transection of the carotid sinus nerve curtailed the changes in the K_m forms of PDE and blocked the increase in RNA-synthesis.

Proposed Course of Project: The following experimental approach will be undertaken to gain better understanding on the mechanism of the chemosensory response of the CB. (1) Studies on first messenger content in CB: The role of various types of nerves innervating the CB in the regulation of DA and NE content during hypoxia will be studied. Measurements of the turnover rate of DA will allow us to establish whether the decrease in DA concentration is caused by enhanced release or by blockade of neurotransmitter synthesis. The use of cholinergic or dopaminergic receptor agonists or antagonists are planned to reveal the nature of the receptor participating in the chemosensory response. (2) Studies on participation of Ca^{2+} binding activator protein in the synaptic communication between carotid sinus nerve terminals and glomus cells. (3) Phosphorylation of synaptosomal membrane protein and nuclear protein fractions in CB during exposure to hypoxic conditions.

Publications:

1. Hanbauer, I.: Mechanisms for tyrosine hydroxylase induction in sympathetic ganglia and carotid body. In Costa, E., Giacobini and Paoletti, R. (Eds.): Advances in Biochemical Psychopharmacology. New York, Raven Press, 1976, Vol. 15, pp. 475-489.

2. Hellstrom, S., Hanbauer, I. and Costa, E.: Selective decrease of dopamine content in rat carotid body during exposure to hypoxic conditions. Brain Res. 118: 352-355, 1976.

3. Hanbauer, I., Lovenberg, W. and Costa, E.: Induction of tyrosine-3-monooxygenase in carotid body of rats exposed to hypoxic conditions. Neuropharmacology 16: 277-282, 1977.

4. Hanbauer, I.: Regulation of tyrosine hydroxylase in carotid body. In Costa, E. and Gessa, G.L. (Eds.): Advances in Biochemical Psychopharmacology. New York, Raven Press, 1977, Vol. 16, pp. 275-280.

5. Hanbauer, I.: The molecular biology of chemoreceptor function: Induction of tyrosine hydroxylase in rat carotid body elicited by hypoxia. In Acker, H., Fidone, S., Pallat, D., Eyzaguirre, C., Lubbers, D.W., and Torrance, R.W. (Eds.): Function and Functional Significance of the Carotid Body. Berlin, Springer Verlag, 1977, In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01847-03 HE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Functional Role of Second Messengers in the Regulation of Neurotransmitter 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	Staff Fellow	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The regulation of phosphodiesterase (PDE) in rat carotid body was studied in response to chemosensory stimulation. Sufficient exposure to hypoxia causes an increase of Ca^{2+} binding protein in the cytosol fraction of carotid body homogenates. PDE occurs in two kinetic forms-one with a high K_m and the other with a low K_m for cAMP. After exposure to hypoxia only a low K_m form of PDE could be detected, indicating an activation of the enzyme. This activation of PDE was prevented by carotid sinus nerve transection prior to hypoxia or by the addition of EGTA to the incubation media.

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Objectives: Cyclic nucleotides have been shown to participate in a multitude of biological processes. As second messengers they mediate the action of most biogenic amines and polypeptide hormones; they also play a role in processes such as cell division, smooth muscle tone, cardiac contractility etc. The steady state concentration of cyclic nucleotides is determined by changes in the activity of nucleotide cyclases or cyclic nucleotide phosphodiesterases (PDE). Both enzyme types are regulated by a common Ca^{2+} binding activator protein. The hydrolysis of cyclic-nucleotides is regulated entirely by PDE. PDE exists in various isozymic forms, which differ in their molecular weight and in their affinity to the various cyclic nucleotides. In several tissues two enzyme forms with a low-and high-K_m for cAMP have been described. One of the isozymic forms which has a high K_m for cAMP can be activated by the Ca^{2+} binding protein and shift to a low K_m-form for cAMP.

It is known that endogenous Ca^{2+} binding protein is stored in synaptic membranes from there it can be released by a mechanism involving cAMP dependent phosphorylation. Since it participates in the regulation of both membrane bound adenylate cyclase and cytosolic PDE, it can be assumed that the activator protein participates in the regulation of the sensitivity of both enzymes to neurotransmitter stimulation. We are using this working hypothesis as a model for studying the possible function of the Ca^{2+} binding protein in transmitter mediated drug effects and in the response to physiologic receptor stimulation specifically the chemosensory response of the carotid body.

Methods: PDE_2 activity was measured with cAMP or cGMP as substrate in presence of 0.01 mM Ca^{2+} and 1 mM Mg. The 5'-nucleotides were hydrolyzed to the corresponding nucleosides by incubating with 5'-nucleotidase contained in snake venom. The nucleosides were separated on aluminum columns equilibrated with 0.1 M NH_4-acetate buffer pH 4.0. The apparent V_{max} and K_m of PDE was measured with either cAMP or cGMP.

The concentration of Ca^{2+} binding activator protein was determined in extracts of heat-treated tissues (76°C for 2 minutes). An aliquot of the 18,000 rpm supernatant was added to activator deficient, high-K_m PDE purified from rat brain and the changes in V_{max} and K_m of the enzyme were measured.

The content of cAMP and cGMP were measured by a radioimmunochemical technique.

Major Findings: During normoxic conditions PDE in rat carotid body occurs in two kinetic forms with a high and low K_m for cAMP. After exposure to hypoxia only a single low K_m-form for $cAMP^m$ can be detected. This increase in affinity of PDE for $cAMP^m$ is Ca^{2+} dependent and fails to occur after CSN-transection. Hypoxia fails to change the K_m of PDE for cGMP, but slightly increases its V_{max} value. Exposure to hypoxia releases a thermostable protein from carotid body-membranes which activates purified high K_m PDE in presence of Ca^{2+}. In order to establish some of the molecular mechanisms that precede the release of the Ca^{2+} dependent activator we studied this

event in vitro. Ca^{2+} cAMP dependent phosphorylation of carotid body homogenates releases the Ca^{2+} binding protein into the cytosol. When tested against purified activator-free PDE it elicits an activation of the enzyme, which is prevented in presence of EGTA.

Proposed Course of Project: In our studies on the functional role of second messengers the regulation of neurotransmitter responses we will persue the following course:

1. Isolation and purification of the Ca^{2+} binding protein.

2. Preparation of antibodies directed against Ca^{2+} binding protein which will be used for turnover studies of this protein but also will allow its direct measurement.

3. Binding studies of various drugs to the Ca^{2+} binding protein will be followed up to help to reveal the action mechanism of these changes.

4. Studies on the regulation of adenylate cyclase, PDE and Ca^{2+} binding protein will be followed up in the heart of normotensive and hypertensive rats.

Publications:

1. Hanbauer, I. and Lovenberg, W.: Presence of a calcium^{2+} dependent activator of cyclic-nucleotide phosphodiesterase in rat carotid body: Effects of hypoxia. Neuroscience, 1977 (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01848-03 HE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Dopamine-β-hydroxylase in Human Cerebrospinal Fluid

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

PI:	Pauline Lerner	Research Chemist	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem.Pharm.	HE NHLBI
	Frederick Goodwin	Chief, Lab. Clin. Psychobiol.	LCP NIMH
	Robert Post	Chief, Sect. Psychobiol.	BPB NIMH
	Daniel van Kammen	Chief, Sect. Neuropsychopharmacol.	CNB NIMH
	L. Frank Major	Clinical Associate	CNB NIMH
	Gerald L. Brown	Medical Officer	BPB NIMH

COOPERATING UNITS (if any)
Biological Psychiatry Branch, NIMH; National Naval Medical Center, Bethesda, Md.; Laboratory of Clinical Neuropharmacology, NIMH

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.9	0.7	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)

We have measured dopamine-β-hydroxylase (DBH) in cerebrospinal fluid (CSF) from psychiatric patients. DBH is the enzyme which catalyzes the conversion of dopamine to norepinephrine; its level in CSF may be an index of central noradrenergic activity. No significant differences have been found among the mean CSF DBH values for 4 diagnostic groups-- primary affective disorder, schizophrenia/schizoaffective disorder, alcoholism, and personality disorders. Preliminary evidence indicates that DBH activity in CSF may be related to mood state and to treatment with psychoactive drugs.

Objectives: We are interested in studying the activity of dopamine-β-hydroxylase (DBH) in the human central nervous system in patients with psychiatric disorders. DBH is found in noradrenergic neurons, and is released from its neuronal storage sites, along with norepinephrine, when the neurons fire. For these reasons, we feel that DBH activity in cerebrospinal fluid (CSF) would be a useful indicator of central noradrenergic activity. It has been suggested, on the basis of DBH assays of human brains at autopsy, that brain DBH is low in schizophrenics. Two different groups have reported conflicting results on this point, and it is possible that technical difficulties arising from the instability of post mortem samples are responsible for the discrepancy. We have, therefore, undertaken to measure DBH in CSF from psychiatric patients and to try to correlate DBH activity with clinical symptoms and drug treatment.

Methods: Cerebrospinal fluid is taken from psychiatric patients who have been hospitalized for schizophrenia, affective illness, alcoholism, or personality disorders. Because the concentration of DBH in CSF is extremely low, a very sensitive assay is required. We have modified a previously existing radioenzymatic assay method by changing the incubation conditions and by using ^{14}C labeled substrate of very high specific activity. This assay is sensitive enough to reproducibly detect and quantitate the very small amount of DBH present in CSF.

Major Findings: DBH has been analyzed in CSF from approximately 70 psychiatric patients. There was no significant difference between mean DBH activities for males and females. There was a small, but statistically significant, correlation between DBH activity in CSF and patient's age. The mean DBH activities for patients from 4 diagnostic groups--primary affective disorder, schizophrenia/schizoaffective disorder, alcoholism, and personality disorders--did not differ significantly. When the patients with affective illness were divided into 2 groups, unipolars and bipolars, their DBH levels were not significantly different. DBH activity in manic patients appeared to be lower than in bipolar patients in the euthymic or depressed states. Preliminary evidence suggests that drugs which increase or decrease the firing rate of catecholaminergic neurons cause parallel changes in the activity of DBH in CSF.

Significance to Biomedical Research and Institute Program: The measurement of DBH activity in CSF appears to be a useful technique for obtaining information on central noradrenergic activity in human subjects. Analysis of DBH in CSF of psychiatric patients should help to elucidate the role of brain norepinephrine in mental illness.

Proposed Course of Project: We plan to expand our study to include larger numbers of patients, since the validity of our conclusions will depend, in part, on the number of subjects studied. We also intend to investigate further the effects of drug treatment and clinical state on DBH in CSF. In addition, we plan to investigate possible correlations between DBH and amine metabolites in CSF.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01850-07 HE

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Biochemistry of the Spontaneously Hypertensive Rats

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

PI:	A. Nagaoka	Visiting Scientist	HE NHLBI
OTHER:	W. Lovenberg	Chief, Sec. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	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)

Three important enzymes required for brain monoamine neurotransmitter synthesis are tyrosine hydroxylase, tryptophan hydroxylase and dopamine-β-hydroxylase. These three enzymes were measured as a function of age in various brain regions in spontaneously hypertensive rats (SHR) and stroke prone substrain (SHR-SP), and control Wistar-Kyoto rats (WKR). There appear to be transient increases in the content of tyrosine hydroxylase in the striatum and hypothalamus of the SHR and SHR-SP between 3 and 8 weeks of age. Likewise there are small increases in dopamine-β-hydroxylase in the hypothalamus, thalamus and pons-medulla. The most significant increase appears to be in the hypothalamus at 5 weeks of age. Changes in hypothalamic dopamine-β-hydroxylase appear to correlate well with the tendency to develop hypertension. Small increases in hypothalamic tryptophan hydroxylase are also seen in the SHR and SHR-SP. Because of the early and transient nature of the changes in brain neurotransmitter synthetic enzymes it is suggested that malfunction of one or more of the brain monoamine systems may be an early event in the development of hypertension in these rat models.

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Objectives: Extensive evidence from studies on metabolism of biogenic amines or activity of the related enzymes indicates that catecholaminergic and serotonergic nerves may play an important role in the development of experimental hypertension. Although we have also studied the central neural mechanisms in the spontaneously hypertensive rats (SHR), the details (nature and extent) of their participation has not yet been established. Recently, a stroke-prone strain of the SHR was introduced to NIH from Japan. The stroke-prone SHR have much more rapid and greater elevation of blood pressure as compared with the regular SHR, which have been used as a model of human essential hypertension for the last decade. The objective of the current experiments is to reexamine some of the neural factors that may participate in the development of hypertension by comparing the stroke-prone SHR, regular SHR and the control Wistar-Kyota rats (WKR).

Methods Employed: Male SHR, stroke-prone SHR and WKR used in these studies were obtained from the Animal Production Section of the NIH. For comparative purposes, two types of experimental hypertension were produced in male WKR. One of them was mineralocorticoid (DOCA) and salt hypertension and the other was renal hypertension.

The activities of monoamine biosynthetic enzymes were measure in brain regions of these kinds of hypertensive rats at various ages. Tyrosine hydroxylase and dopamine-β-hydroxylase (DBH) activities were assayed using sensitive radio-enzymic methods. Tryptophan hydroxylase was measured by a spectrofluorometric method. In the periphery, plasma DBH activity and norepinephrine were examined. Systolic blood pressure was measured the day before the sacrifice by a tail-cuff method.

The statistical significance was determined by an unpaired Student's t-test, and a probability of less than 0.05 was considered significant.

Major Findings: The major findings of this project were reported in the previous annual report.

Significance to Biomedical Research and the Program of the Institute: There is increasing evidence that the central nervous system plays a major role in the development and/or maintenance of increased blood pressure in essential hypertension. It was therefore of interest to examine the key biosynthetic enzymes for dopamine, norepinephrine and serotonin. The content of these enzymes in specific brain regions is thought to be indicative of the function of the aminergic neuronal systems of the brain. The current studies are consistent with our general hypothesis that early alteration in certain neuronal systems of brain result in increased outflow of impulses to the vasculature which in turn increase the rate of contractile protein synthesis in the small resistance vessels.

Proposed Course of Project: Recent studies in our laboratory suggest the synthesis of catecholamine neurotransmitters in brain is regulated primarily by changes in the kinetic state of tyrosine hydroxylase. This regulation may be realized by a cyclic AMP-dependent protein phosphorylation. We propose to study the regulatory properties of tyrosine hydroxylase in the hypertensive

rat models and to determine what effects antihypertensive druge have on the kinetic state of tyrosine hydroxylase.

Publications:

Nagaoka, A. and Lovenberg, W.: Plasma norepinephrine and dopamine-β-hydroxylase in genetic hypertensive rats. Life Sci. 19: 29-34, 1976.

Nagaoka, A. and Lovenberg, W.: Regional changes in the activities of aminergic biosynthetic enzymes in the brains of hypertensive rats. European J. of Pharmacology 43: 297-306, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HL 01851-03 HE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Regulation of Tyrosine Hydroxylase in the Central Nervous System

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

PI:	Matthew M. Ames	Research Associate	HE NHLBI
OTHER:	Pauline Lerner	Postdoctorate 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 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	0.8	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)

The primary goal of these studies is an understanding of the short-term regulation of tyrosine hydroxylase in the central nervous system. We have previously shown an activation of the soluble enzyme (via a decrease in the apparent K_m for the reduced pterin cofactor) by phosphorylation or catecholamine removal by G-25 chromatography in striatum and adrenal gland. We have extended these observations to the hypothalamus. Our major finding has been that phosphorylation results in a significant reduction in K_i for dopamine and release from end-product inhibition as its primary means of tyrosine hydroxylase activation. The data suggests that this mechanism could be operative in vivo at physiolcgical concentrations of dopamine.

695

PHS-6040

Objectives: As the rate-limiting enzyme in catecholamine biosynthesis in the central nervous system, tyrosine hydroxylase is intimately involved in the regulation of neurons utilizing catecholamine neurotransmitters. In previous reports, we have described *in vitro* activation of the enzyme by phosphorylation, catecholamine removal, and addition of polyanions to our preparations. We also found that calcium and EGTA did not activate the enzyme. The objective of our continued studies have been to better describe the mechanism of activation by phosphorylation, and to investigate the relationship between phosphorylation and end-product inhibition of tyrosine hydroxylase. This is of particular interest in that these two regulatory mechanisms are thought to be of importance in the *in vivo* control of tyrosine hydroxylase.

Methods: Tissues were dissected from male Sprague-Dawley rats by standard procedures. The enzyme was assayed by the tritium release method using the chemical (2-mercaptoethanol) reducing system. Catecholamines were removed by either Sephadex G-25 on cation-exchange (Dowex 50) chromatography. K_i values were determined using the Dixon plot method, K_m values by the Lineweaver-Burke plot method.

Major Findings: Work described in previous reports demonstrated activation of tyrosine hydroxylase by addition of phosphorylation components to a 40,000 x g supernatant fraction as well as by catecholamine removal (G-25 chromatography of 40,000 x g supernatant). The activation is rat striatum, hypothalamus, or adrenal was characterized by a reduction in the apparent K_m for the reduced pterin cofactor, 6-methyltetrahydropterin ($\sim$ 0.5 mM to $\sim$ 0.15 mM). In further studies, it was found that phosphorylation of tissue extracts already subjected to G-25 chromatography (and thus free of >90% endogenous catecholamines) did not result in significant further activation.

Recent work has now shown that catecholamines may also be efficiently removed by filtration of tissue extracts through small Dowex 50 cation-exchange columns, affording a quick method for this manipulation which results in almost no dilution of protein concentration. As with the G-25 chromatography, the apparent K_m is significantly reduced after this treatment. Further, such preparations are not significantly activated by phosphorylation, also analogous to previous results.

In an attempt to clarify these findings, the K_i for dopamine was determined for crude striatal extracts, filtered extracts (G-25 and Dowex 50), and phosphorylated extracts. Filtration by either method did not raise the K_i value ($\sim 1 \times 10^{-5}$M), whereas phosphorylation resulted in a significant increase in the K_i ($\sim 3.5 \times 10^{-5}$M). At physiological concentrations of dopamine, such a change in the effect of dopamine could effectively release tyrosine hydroxylase from end-product inhibition. This was verified by addition of 3×10^{-6}M dopamine to crude, filtered, or phosphorylated tissue extracts. Only in the latter preparation did the dopamine have no effect on the activity of the enzyme, showing release from catecholamine end-product inhibition.

Significance to Biomedical Research and Institute Program: The key elements in control of tyrosine hydroxylase in vivo appear to be the low cofactor concentration, and catecholamine end-product inhibition which is competitive in regard to the cofactor. Phosphorylation lowers the apparent K_m for the cofactor, and according to our findings, releases the enzyme from end-product inhibition. This phosphorylation may be of real significance in the regulation of this important neural enzyme, representing a fast-acting regulatory mechanism in the presence of normal catecholamine concentrations in the neurons. Such regulation may be important in disease states with central nervous system involvement.

Proposed Course of Project: The phosphorylation of tyrosine hydroxylase will be further studied, in conjunction with other important regulatory mechanisms. These studies will be directed towards a better understanding of the inter-relationships between the various methods of tyrosine hydroxylase activation.

Publications:

1. Lovenberg, W., Ames, M.M. and Lerner, P.: Regulation of tyrosine hydroxylase activity. In Costa, E. and Gessa, G.L. (Eds.): Advances in Biochemical Psychopharmacology. New York, Raven Press, 1977, Vol. 16 pp. 461-464.

2. Lerner, P., Ames, M.M. and Lovenberg, W.: The effect of EGTA and calcium on tyrosine hydroxylase activity. Molecular Pharmacology 13: 44-49, 1977.

3. Lovenberg, W., Ames, M.M. and Lerner, P.: Mechanisms of acute regulation of tyrosine hydroxylase. In: Psychopharmacology: A Generation of Progress, Lipton, M.A., DiMascio, A. and Killiam, K. (Eds.) Raven Press, N.Y. In press (1977).

4. Lerner, P., Hartman, P., Ames, M.M. and Lovenberg, W.: The role of reductants in the tyrosine hydroxylase assay. Archives Biochem. & Biophysics. In press (1977).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01852-06 HE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Regulation of Hydroxyindole Pathway in the Pineal Gland

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

PI:	J.J. Morrissey	Staff Fellow	HE NHLBI
OTHER:	W. Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharamcology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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 scope of this project is to define the molecular events involved in the neuronal induction of serotonin-N-acetyltransferase in the pineal gland. During the course of this investigation it was found that there seemed to be no significant change in gross RNA synthesis, however, general protein synthesis was uniformly elevated approximately 20%. Immediately upon β-agonist stimulation of pineal gland adenylate cyclase, and cyclic AMP content were elevated and a nuclear protein was phosphorylated. This protein remained phosphorylated during the actinomycin D sensitive phase of enzyme induction. Approximately 70% of the poly(A) containing RNA synthesis of the pineal gland could be inhibited by actinomycin D or α-amanitin without affecting enzyme induction.

Objectives: The hydroxyindole pathway of the pineal gland offers a unique opportunity to examine mammalian enzyme regulation and regulation of protein synthesis. The synthesis of melatonin is controlled by the levels of serotonin-N-acetyltransferase (NAT) which in turn is under neuronal control. Beta receptor stimulation causes a 50-fold increase in NAT activity. Cyclic AMP is the second messenger in this system. The objective of this project is to define at a molecular level the mechanism by which cAMP is eliciting the large increase in NAT activity.

Methods: The control of serotonin-N-acetyltransferase (NAT) was studied in the culture rat pineal. Levels of the enzyme were measured in individual glands by a sensitive radiochemical technique. RNA synthesis was assayed by the incorporation of ^{3}H-uridine into cold TCA precipitable material in post-mitchondrial supernatants of pineal homogenates. The radiolabeled RNA was further characterized by phenol extraction and agarose gel electrophoresis. Various inhibitors of RNA synthesis were used to study the effect on the induction of NAT. Nuclei from cultured rat pineal glands were isolated by conventional methods. For experiments in which the incorporation of $^{33}PO_4$ into nucleoproteins, the proteins were extracted and characterized by SDS gel electrophoresis by autoradiography.

Protein synthesis in the cultured pineals was measured by the incorporation of ^{3}H or ^{14}C-labeled amino acids or ^{35}S-methionine into hot TCA precipitable proteins of post-mitochondrial supernatants. The radiolabeled protein products were characterized by SDS (sodium dodecyl sulfate) disc gel or SDS-slab gel electrophoresis and liquid scintillation counting or autoradiography. Phosphoprotein synthesis was assayed by the incorporation of ^{33}P-phosphate into cultured pineal glands. The post-mitochondrial supernatants were separated by SDS-slab gel electrophoresis and autoradiographed.

Protein kinase activity was also measured in cultured pineal glands at various times during the isoproterenol stimulated induction of NAT activity. The kinase was measured by the incorporation of ^{32}P into histone Type II-A using α-^{32}P-ATP and millipore filtration.

Major Findings: The RNA synthesis inhibitors actinomycin D and α-amanitin were found to inhibit the synthesis of poly(A) containing RNA by 70% without affecting the induction of NAT by isoproterenol. In the range of 80-85% inhibition of poly(A) containing RNA synthesis, both inhibitors did markedly diminish enzyme induction. An 85% inhibition of poly(A) containing RNA synthesis by cordycepin did not, however, affect the induction process. An inhibition dose of actinomycin D was only effective on inhibiting induction if administered during the first 3 hours of induction. (This is at a time when enzyme activity has not yet increased.) During this initial 3 hour period, when apparent RNA synthesis is required, a 34,000 dalton nuclear protein is phosphorylated. This protein is not phosphorylated during the last 1.5 hours of enzyme induction (an actinomycin D insensitive phase).

Throughout the entire 6 hours of NAT induction by ß-agonists there is a uniform increase in the incorporation of radioactive amino acids into protein.

This increase is also seen with dibutyryl cyclic AMP. This increased incorporation is probably due to an increase in protein synthesis since there is no significant change in the uptake of radioactive amino acids into the cultured glands in the presence of isoproterenol or dibutyryl cyclic AMP. The increase in protein synthesis is probably a general increase in the synthesis of all pineal gland proteins since autoradiographic analysis of labeled proteins separated by SDS gel electrophoresis revealed no significant change in the number of proteins synthesized. This increase in protein synthesis by the β-agonist isoproterenol was blocked by low concentrations of the β-antagonist propranolol. The increased protein synthesis was not dependent on new RNA synthesis since an inhibiting dose of actinomycin D did not affect the incorporation of labeled amino acids into protein.

As found in the previous report, pineal protein kinase activity was elevated in isoproterenol treated pineal glands at an early time (10-20 min) during enzyme induction.

Significance to Biomedical Research and Institute Program: It may be concluded from these studies that β-agonists may exert their effect on pineal gland NAT induction as depicted below:

β-agonist → Sympathetic nerve (norepinephrine) → Pineal β-Receptor → Adenyl cyclase → cAMP → Protein kinase → Nuclear protein phosphorylation → Transcriptive activity → Increased translative activity → Elevated NAT activity

Transcriptive activity is inferred due to an actinomycin D sensitivity at early stages of induction but an insensitivity at latter stages. This putative RNA required for enzyme induction may not be polyadenylated because of the cordycepin insensitivity. Translational activity is uniformly elevated throughout the induction process but this may place the gland in a receptive position for synthesis of new proteins. It is not known whether the NAT enzyme is a primary product of induction or if a modifying enzyme is primarily induced which secondarily activates an inactive proenzyme.

Proposed Course of Project: The following studies will be done in an effort to further understand the mechanism by which β stimulation results in an apparent induction of serotonin-N-acetyltransferase .

(a) Further attempts to characterize the specific protein that becomes phosphorylated during induction will be made. Because of the trace amounts of this protein only general characterization will be possible.

(b) We plan to examine the role of messenger RNA in the induction phenomenon directly, by extracting mRNA from induced and non-induced gland and attempting to translate it in a xenopus oocyte system.

Publications:

1. Winters, K., Morrissey, J.J., Loos, P.J. and Lovenberg, W.: Pineal protein phosphorylation during N-acetyltransferase induction. Proc. Natl. Acad. Sci. 74: 1928-1931, 1977.

2. Morrissey, J.J. and Lovenberg, W.: The synthesis of RNA in the pineal gland during N-acetyltransferase induction. Biochem. Pharmacol. In press (1977).

3. Morrissey, J.J. and Lovenberg, W.: The synthesis of RNA in the pineal gland during N-acetyltransferase induction: The effects of actinomycin D, α-amanitin and cordycepin. Biochem. Pharmacol. In press (1977).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01854-06 HE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Characterization of Human and Bovine Dopamine-β-hydroxylase

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

PI:	Robert C. Rosenberg	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 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.6	

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 active species of dopamine-β-hydroxylase (DBH) in whole human serum have been identified and characterized.

PHS-6040

Objectives: The goal of this project has been to study the molecular properties of the species of DBH found in human serum or plasma.

Methods: Gradient ultracentrifugation and gel filtration have been used to study the molecular properties of the multiple forms of DBH found in human plasma or serum.

Major Findings: The molecular properties of normal human serum dopamine-β-hydroxylase (DBH) have been studied. Two active species of DBH have been found in normal human serum. An average of 78% of the total activity is associated with the larger of these species (Fract. I) while the remainder of the activity is associated with a smaller species (Fract. II). No interconversion of active enzyme is observed between these species at the pH and ionic strength of serum. The molecular weights of these two species have been determined from independent measurements of their sedimentation coefficients and Stokes radii. The principle species of DBH in human serum (Fract. I) was found to have a molecular weight of 289,000. Its molecular weight, sedimentation coefficient, and Stokes radius are very similar to the corresponding parameters found for bovine DBH under the same conditions. The minor species of DBH in serum (Fract. II) has a molecular weight of 147,000. Thus, Fract. I is very likely a dimer of Fract. II. By analogy to the subunit structure of both bovine DBH and Fract. I of human serum DBH, it is concluded that Fract.II is an active dimer of the basic subunit of human DBH. Both Fract. I and Fract. II appear to be highly assymetric in solution based on the calculated values of their frictional ratios.

Significance to Biomedical Research and Institute Program: DBH is essential for the function of adrenergic neurons and chromaffin cells since it catalyzes the final step in the synthesis of the neurotransmitter, norepinephrine. DBH has been found to be localized within the synaptic and chromaffin granule vesicles, and upon nerve stimulation it is released along with the neurotransmitter by an exocytotic process. Hyperactivity of the sympathetic nervous system has been implicated in the development or maintenance of essential hypertension in man and experimental forms of hypertension in animals. Serum levels of DBH have been used as an index of sympathetic activity, although there is considerable controversy as to whether this approach is valid. Detailed knowledge at the molecular level of the structure, function and regulation of DBH is important for a complete understanding of the functioning of the adrenergic nervous system in both normal and disease states.

Proposed Course of Project: This project is to be discontinued.

Publications:

1. Rosenberg, R.C. and Lovenberg, W.: Active dimers of dopamine-β-hydroxylase in human serum. Molecular Pharmacology, In press (1977).

2. Kopin, I.J., Kaufman, S., Viveros, H., Jacobowitz, D., Lake, C.R., Ziegler, M.G., Lovenberg, W., and Goodwin, F.K.: Dopamine-β-hydroxylase: Basic and clinical studies. Combined Clinical Staff Conference at the National Institutes of Health. Ann. Int. Med. 85: 211-223, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01859-06 HE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Characterization and Mechanism of Action of Dopamine-β-hydroxylase
(Revised Title)

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

PI:	R.C. Rosenberg	Staff Fellow	HE NHLBI
OTHER:	G.A. Walker	NIH Postdoctorate Fellow	HE NHLBI
	W. Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI
	H. Kon	Research Chemist	LCP NIAMDD
	J. Gimble	Chemist	HE NHLBI

COOPERATING UNITS (if any)

NIAMDD, Laboratory of Chemical Physics

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.6	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 long-term objective of this project is to characterize and understand the mechanism of action and regulation of dopamine-β-hydroxylase (DBH). The immediate goals have been to measure the redox potential of DBH and to study the effects of substrates and products on the active site of the enzyme. The effect of $Fe(CN)_6^{4-}$ as an electron donor for DBH has also been studied.

Objectives: The objective of this project is to characterize and understand the mechanism of action of dopamine-β-hydroxylase.

Methods: DBH was isolated and purified from bovine adrenal medulla. In the redox studies hydroquinone and ferricynide were used as mediators. Electron paramagnetic resonance spectroscopy was used to monitor the oxidation state of the copper present in DBH as well as to detect the effects of substrates and products on the copper center. Steady state kinetic techniques were used to study the mechanism of the DBH catalized reaction with ferrocyide as the electron donor.

Major Findings: All the chemically detectable copper in DBH (4 gm-atom per tetramer) was found to be epr detectable in the oxidized form of the enzyme. The enzyme could be completely reduced or oxidized by ascorbate or ferricynide, respectively. Equilibria between the protein bound copper and the hydroquinone-quinone couple have indicated that the midpoint potential for DBH at pH 7.0 is +307 ± 12 mV. The effect of substrate and products on the epr spectrum of the enzyme was also investigated. In the presence of substrates and products with a 4-hydroxyl group in the aromatic ring, superhyperfine structure in the g region of the epr spectrum was observed which is attributable to nitrogen ligation of the copper center. The enzyme as isolated was found to be partially reduced. Complete deoxygenation of the resting enzyme, however, produced a decrease in the amplitude of the epr signal-indicating further reduction of the copper center. This effect can be explained by postulating the existence of a reduced enzyme-oxygen complex.

We have also studied the mechanism of the DBH catalyzed reaction using $Fe(CN)_6^{4-}$ as the electron donor instead of ascorbate. We have found that $Fe(CN)_6^{4-}$ can inhibit the enzyme at high concentrations thus explaining why previously, $Fe(CN)_6^{4-}$ was not considered to be a good electron donor to DBH. Below the inhibitory range, however, our kinetic studies indicate that $Fe(CN)_6^{4-}$ is a good electron donor to DBH, giving a V_{max} that is comparable to the value found with ascorbate. However with $Fe(CN)_6^{4-}$ as the electron donor the reaction catalyzed by DBH follows a sequential mechanism. We have also found that using $Fe(CN)_6^{4-}$ as the sole electron donor for the DBH catalyzed reaction obviated the need for catalase in the reaction mixtures. Copper ion has been found to have a complex effect on the rate of the DBH catalyzed reaction when $Fe(CN)_6^{4-}$ is the sole electron donor. It can reverse the inhibitory effects of high concentration of $Fe(CN)_6^{4-}$. However, at sub-inhibitory concentrations of $Fe(CN)_6^{4-}$, copper appears to act as competitive inhibitor of DBH with respect to $Fe(CN)_6^{4-}$. $Fe(CN)_6^{4-}$ was also found to inhibit the ascorbate mediated DBH catalyzed conversion of tyramine to octopamine. $Fe(CN)_6^{4-}$ was found to give uncompetitive type inhibition with respect to ascorbate but mixed type inhibition with respect to tyramine. These results indicate that the inhibitory action of $Fe(CN)_6^{4-}$ occurs at a site other than the electron accepting site on the enzyme.

Significance to Biomedical Research and Institute Program; DBH is essential for the function of adrenergic neurons and chromaffin cells since it catalyzes the final step in the synthesis of the neurotransmitter, norepinephrine. DBH

has been found within the synaptic and chromaffin granule vesicles, and upon nerve stimulation it is released along with the neurotransmitter by an exocytotic process. Hyperactivity of the sympathetic nervous system has been implicated in the development or maintenance of essential hypertension in man and experimental forms of hypertension in animals. Detailed molecular knowledge of the structure, function and regulation of DBH is important for a complete understanding of the functioning of the adrenergic nervous system in both normal and disease states. There is a large body of work on the in vivo inhibition of DBH by various chelating and thiol compounds, some of which have been used as antihypertensive agents in man. A detailed understanding of the mechanism by which these inhibitors modulate DBH activity can provide valuable information on the functioning of the native enzyme as well as suggestions for potentially useful new antihypertensive agents.

Proposed Course of Project: Studies using $^{17}O_2$ enriched oxygen will be persued in order to characterize the nature of the reduced enzyme-oxygen complex. Titrations of the enzyme with irreversible inhibitors such as CO and H_2O_2 will be undertaken in order to determine the number of active sites in the enzyme tetramer.

Publications:

1. Walker, G.A., Kon, Hidea and Lovenberg, W.: An investigation of the copper site(s) of dopamine-β-hydroxylase by electron paramagnetic resonance. Biochimica et Biophysica Acta 482: 309-322, 1977.

2. Kafka, M.S., Blumenthal, R., Walker, G. and Pollard, H.: The effect of dopamine-β-hydroxylase on the electrical conductance of biomolecular lipid membranes. Membrane Biochem. In press (1977).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01861-05 HE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Receptor Mediated Feedback Control of Brain Tryptophan Hydroxylase Activity (Revised Title)

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

PI:	Donald M. Kuhn	NIH Postdoctorate 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 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	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 activity of tryptophan hydroxylase, the rate-limiting enzyme in the synthesis of the neurotransmitter serotonin, was determined in extracts of various rat brain structures after treatments which increase or decrease the availability of serotonin in the synaptic cleft. Drugs promoting the rapid release of serotonin [e.g., p-chloroamphetamine (PCA) and p-chloromethamphetamine (PCMA)] produce an acute reduction (onset by 2-3 hours) in the activity of tryptophan hydroxylase in brain areas rich in serotonin nerve endings. In preliminary experiments, inhibition of the serotonin releasing effects of PCMA by chlorimpramine blocked the reduction in enzyme activity. While it is usually hypothesized that PCA itself is taken up into the presynaptic bouton where it inhibits tryptophan hydroxylase, this project will examine the possibility that 5-HT released into functional activity (i.e., onto the receptor) initiates a sequence of neuronal and biochemical events which has as its end point, a rapid compensatory decrease in serotonin synthesis at the rate limiting hydroxylase step.

Objectives: The enzyme tryptophan hydroxylase (TPH; EC 1.14.16.4) is interesting and important in studies of brain function since it is the rate limiting enzyme in the biosynthesis of the neurotransmitter serotonin (5-HT). Thus, treatments which alter the activity of TPH may determine the amount of 5-HT actually available for synaptic release. However, apart from the well-known effects that administration of the substrate tryptophan has on 5-HT synthesis, little is known about the in vivo regulation of the activity of TPH. It is the objective of this study to learn more about TPH by examining the enzymic response to alterations in 5-HT receptor status. PCA, PCMA, and possibly fenfluramine, induce acute reductions in TPH activity primarily in brain areas rich in 5-HT nerve endings (hippocampus, striatum, septum). It has been concluded that these drugs are taken up into the presynaptic nerve ending where they exert a local neurotoxic effect. Indeed, inhibition of the 5-HT uptake process with chlorimipramine (CMI) blocked the PCMA reduction of TPH by some local neurotoxic effect. While attractive, this "toxic" hypothesis cannot completely explain the phasic recovery seen in TPH activity after the initial rapid decrease. This recovery takes place too soon after drug administration to be accounted for by an induction of TPH (i.e., synthesis and transport of new enzyme protein). Perhaps a more basic explanation would include a receptor mediated feedback mechanism: PCA causes a rapid and extensive release of 5-HT into functional activity and, as a compensation for increased receptor activation, TPH activity is decreased. The CMI antagonism of PCA induced reductions in TPH is not inconsistent with this reasoning: CMI also blocks the ability of PCA to release 5-HT. The following studies will be carried out to examine this hypothesis.

Methods: (1) Effects of increased 5-HT receptor activation on TPH: Drugs used will include directly acting 5-HT agonists (LSD, 5-HT, 5,7-DHT, quipagine) and "indirect" (release 5-HT) agonists (fenfluramine, 5-HT, CMI). Agents which block 5-HT receptors (e.g., methiothepin, methysergide) will be tested as antagonists of the drugs listed above. (2) Effects of decreased 5-HT receptor activation on TPH activity: Methiothepin and methysergide will be injected by themselves and the enzyme response to receptor blockade will be determined.

TPH will be assayed in rat brain extracts by the method of Baumgarten et al. (J. Neurochem. 21: 251-253, 1973). 5-HT and 5,7-DHT will be injected intraventricularly. All other drugs will be injected intraperitoneally.

Significance to Biomedical Research and Institute:

Program: Very little is known about the acute in vivo regulation of tryptophan hydroxylase activity. Based on the numerous behavioral (e.g., sleep, food intake) and pathological states (e.g., carcinoid syndrome) involving 5-HT, a clearer understanding of TPH regulation will certainly help elucidate the role of this neurotransmitter in normal and altered states of brain function. Furthermore, the mechanisms of action of several pharmacological agents which one thought to act on the 5-HT system (e.g., LDS, lithium) can be more completely understood by determining how they influence TPH activity.

Proposed Course of Project: A different strategy will be employed to evaluate the concept of receptor mediated changes in enzyme activity. Many of the drugs listed in the Methods section usually have numerous effects on the 5-HT system. For example, CMI apparently blocks the uptake of PCMA and it also blocks the PCMA induced release of 5-HT. Further, 5-HT antagonists (in general) block 5-HT release in addition to blocking the receptors. Thus, methiothepin could block PCMA-induced decreases in TH by preventing the PCMA induced release of 5-HT, by blocking 5-HT receptors, or by a combination of these effects. To avoid the interpretational compexities which may be encountered by studying brain tissue, an intact cell system which contains 5-HT and TPH but which is devoid of neuronal-like postsynaptic receptors will be employed, namely, the mouse mast cell tumor. If PCA is taken up by the mast cell and inhibits tryptophan hydroxylase, the hypothesis of receptor mediated feedback inhibition of TPH can be more accurately assured.

Publications:

1. Lovenberg, W.: Enzyme changes as an index of neurotoxin specificity. Ann. N.Y. Acad. Sci. In press (1977).

2. Baumgarten, H.G., Klemm, H.P., Lachenmayer, L., Björklund, A., Lovenberg, W. and Schlossberger, H.G.: Mode and mechanism of action of neurotoxic indoleamines: A review and progress report. Ann. N.Y. Acad. Sci., In press (1977).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01862-02 HE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Purification of an Enzyme which N-acetylates Serotonin from Rat Liver

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

PI: J.J. Morrissey	Staff Fellow	HE NHLBI
OTHER: W. Lovenberg	Chief, Sect. Biochem., Pharmcol.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.2	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)

Enzymes which are capable of N-acetylating serotonin or tryptamine were partially isolated from rat pineal gland and rat liver. Both enzymes displayed ping-pong kinetics suggestive of an acetylated enzyme intermediate. The molecular weight of the liver enzyme was 26,000 daltons while the pineal gland enzyme was found to be 39,000 daltons. The reagent β-mercaptoethylamine appeared to dissociate both the liver and pineal enzymes to 10,000-12,000 dalton subunits. It is suggested that the active liver enzyme is a dimer of 12,000 dalton subunits while the active pineal enzyme is a tetramer of 10,000 dalton subunits.

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Objectives: The purpose of this investigation is to compare the molecular properties of serotonin-N-acetylating enzymes in the rat liver and the rat pineal gland. The rat pineal enzyme is inducible by β-agonists while the liver enzyme is not inducible. This project is designed to ascertain whether the inducible pineal enzyme is the same as the non-inducible liver enzyme.

Methods: N-acetyltransferase activity was determined by a sensitive radiochemical technique using tryptamine as an acetate acceptor from the acetate donor acetyl coenzyme A. All enzyme assays were performed at a pH of 6.5. The molecular weight of pineal gland and liver N-acetyltransferase was estimated on 0.6 x 25 cm Sephadex 6-100 columns in 50 mM potassium phosphate ± 4 mM β-mercaptoethylamine.

Major Findings: Kinetic analysis of the pineal gland or liver N-acetyltransferase reactions yielded parallel double reciprocal plots characteristic of a ping-pong mechanism. This suggests an acetylated enzyme intermediate in the course of acetate transfer from the donor acetyl coenzyme A to the acceptor tryptamine. The K_m values for the substrates were derived from secondary plots of the primary intercepts and are listed below.

Enzyme Source	K_m Tryptamine	K_m AcetylCo A	Molecular Weight
Liver	240 ± 30 μM	125 ± 20 μM	26,000 ± 1,000
Pineal	530 ± 40 μM	50 ± 10 μM	39,000 ± 3,000

Estimation of active enzyme molecular weights listed above as well as differences in the K_m values for substrates suggests that the inducible pineal gland N-acetyltransferase is a different enzyme from the non-inducible liver enzyme. The reagent β-mercaptoethylamine, which stabilizes the usually labile pineal gland N-acetyltransferase activity was found to partially dissociate the liver and pineal enzymes into 10,000-12,000 dalton subunits during Sephadex G-100 chromatography antibodies to partially purified pineal gland or liver enzymes were not successfully made.

Significance to Biomedical Research and Institute Program: While previous investigations had suggested that the pineal gland and extra pineal N-acetyltransferases were different enzymes, our study of molecular properties unambiguously proves this to be true. The inducible pineal gland enzyme is an unique enzyme under inductive control rather than an enzyme common to many tissues under unique inductive control in the pineal gland.

Proposed Course of Project: No further experiments are planned at this time.

Publications:

1. Morrissey, J.J., Edwards, S.B. and Lovenberg, W.: Comparison of rat pineal gland and rat liver N-acetyltransferase. Biochem. Biophysical. Research Comm. In press (1977).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01863-02 HE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Tryptophan Hydroxylase Activity in Blood Platelets
(Revised Title)

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

PI:	Jeremiah Morrissey	Staff Fellow	HE NHLBI
OTHER:	Margaret Walker	NIH Postdoctoral 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 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	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)

Attempts were made to measure tryptophan hydroxylase activity in blood platelets. Under conditions promoting maximal tryptophan hydroxylation in other tissue extracts (e.g., pineal gland or rat brain) and cultures (pineal), no tryptophan hydroxylase activity was found in platelets isolated from human or rat blood. These results suggest that the serotonin found in platelets is largely accumulated via an active uptake mechanism and cannot be attributed to the hydroxylation of tryptophan to serotonin within this plasma organelle.

Objectives: Tryptophan hydroxylase is the rate-limiting enzyme in the biosynthesis of the neurohumoral agent serotonin. Blood platelets, by virtue of their ability to accumulate, store, and release serotonin, are a functionally important depot for this vasoactive substance. Furthermore, possession of these various "nerve-ending"-like mechanisms makes the platelet a heuristic model for the serotonergic neuron. It is not known, however, whether the large amounts of serotonin found in platelets are more dependent on uptake from plasma or on the de novo synthesis of serotonin from tryptophan within the platelet via the hydroxylase enzyme. Taking advantage of a more sensitive and specific assay for tryptophan hydroxylase, developed in this laboratory, studies were undertaken to determine the extent to which platelets could synthesize serotonin from tryptophan.

Methods: Platelet rich plasma (PRP) was prepared by centrifugation of heparinized whole blood obtained from human volunteers or male Sprague-Dawley rats at 200 g for 15 min. at 4°. The resulting PRP was again centrifuged at 200 g for 15 min. at 4° to remove contaminating leukocytes. Platelets were sedimented at 2500 g for 15 min. at 4° and washed twice in 50 mM Tris-Cl, pH 7.4, 100 mM NaCl, 2 mM dithiothreitol by resuspension-centrifugation. The final platelet pellet was resuspended in a small volume of 50 mM Tris-Cl, pH 7.4, and 2 mM dithiothreitol, sonicated, and centrifuged at 27,000 g for 15 min. at 4°. Resulting supernatant fractions were used for the assay. Rat pineal glands and brain regions were homogenized in 50 mM Tris-Cl, pH 7.4, and 2 mM dithiothreitol and similarly centrifuged.

Tryptophan hydroxylase activity was determined by the method of Friedman et al. (J. Biol. Chem. 247: 4165, 1972) as modified by Baumgarten et al. (J. Neurochem. 21: 251, 1973). Briefly, a 0.05 ml portion of platelet extract or 0.02 ml of pineal, tegmentum, or striatum extract was incubated in a final volume of 0.08 ml containing 12 µmol of Tris-Cl, pH 7.4, 0.4 µmol dithiothreitol, and 17 nmol of 6-methyltetrahydropterin at 37° for 5 min. Reactions were started by the addition of 0.02 ml of 2 mM tryptophan. After 40 min. the reactions were stopped by the addition of 0.02 ml of 6N perchloric acid and centrifuged to remove the precipitated protein. A 0.01 ml portion of the supernatant was mixed with 0.03 ml of concentrated HCl and the fluorescence was measured at an emission wavelength of 540 nM with an excitation wavelength of 290 nM. The amount of 5-hydroxytryptophan formed was determined from a standard curve run simultaneously.

Major Findings: Using assay conditions which favor maximal tryptophan hydroxylation, platelets from both human and rat blood were found to be devoid of tryptophan hydroxylase activity.

Since platelets contain high endogenous serotonin concentrations, it is possible that extensive end-product inhibition of the enzyme precluded its measurement. To control for this, serotonin was added to rat brain extracts (tegmentum). Under these conditions the tryptophan hydroxylase activity was not inhibited, ruling out end-product inhibition.

To eliminate the possibility that an endogenous inhibitor was liberated during the isolation and preparation of platelets, rat tegmental extracts were mixed with platelets and tegmental tryptophan hydroxylase activity was subsequently determined. After this treatment, tegmental hydroxylating activity was neither enhanced nor inhibited.

Using a radioactively labeled substrate, the complete metabolic pathway from ^{3}H-tryptophan to ^{3}H-serotonin was monitored. While cultured pineal gland demonstrated robust tryptophan hydroxylase activity, there was no conversion of ^{3}H-tryptophan to either ^{3}H-5-HTP or ^{3}H-seotonin in human or rat platelet-rich plasma indicating a lack of tryptophan hydroxylase activity in this organelle.

Significance to Biomedical Research and Institute Program: These experiments establish that blood platelets do not possess tryptophan hydroxylase activity. Therefore, the serotonin usually found within platelets is apparently accumulated primarily through an active uptake mechanism. Without appreciable tryptophan hydroxylase activity, the platelet cannot serve as a complete model for the serotonergic nerve ending. In light of these results, however, platelets still function as a storage depot for serotonin and the uptake system may be one of the most important factors modulating free vs stored plasma serotonin levels under normal conditions.

Proposed Course of Project: This project to be terminated.

Publications:

1. Morrissey, J.J., Walker, M.N. and Lovenberg, W.: The absence of tryptophan hydroxylase activity in blood platelets. Proc. Soc. Exp. Biology and Med. 154: 496-499, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01864-02 HE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Effect of Phenelzine on Tryptophan and Tyrosine Hydroxylase Activities

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

PI:	Donald Robinson	Guest Worker (Univ.of Vt., College of Medicine)	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI
	I.C. Campbell	Staff Fellow	LCS NIMH
	D. Murphy	Chief, Sect.Chem. Neuropharm.	CN NIMH

COOPERATING UNITS (if any)
Laboratory of Clinical Science, NIMH

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.6	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)

Following treatment of rats for varying periods of time with the monoamine oxidase inhibitors, phenelzine, tranylcypromine, clorgyline or pargyline, changes in endogenous levels of norepinephrine (NE), dopamine (DA) and 5-hydroxytryptamine (5-HT) have been measured. In addition, regional changes in tyrosine hydroxylase, tryptophan hydroxylase and aromatic L amino acid decarboxylase were assessed.

There are initial increases in whole brain NE, DA and 5-HT levels with a peak between 3-7 days, followed by a gradual decrease towards control levels over the next several weeks. Striatal tyrosine hydroxylase (TH) activity becomes significantly decreased from control levels by the third week of phenelzine and tranylcypromine treatment and remains so at six weeks. TH is unaffected by chronic pargyline or clorgyline treatment. Tryptophan hydroxylase (TpOH) is significantly increased in mesencephalic tegmentum at three and at six weeks of phenelzine treatment. Clorgyline and pargyline did not affect TpOH activity. Decarboxylase was unchanged by phenelzine treatment but increased by tranylcypromine.

Objectives: Most psychotropic drugs are administered for a minimum of weeks to months for therapeutic effect in man, yet little has been published regarding the biochemical pharmacology of these drugs beyond acute treatment of a few days. Thus, we have undertaken the study of chronic MAO inhibitor effects on monoamine systems in rat brain.

The purpose of these studies is to examine TH, TpOH and decarboxylase in various brain regions over a 42 day period to determine if there are compensatory changes in enzyme activity which would account for the initial peak and subsequent drop in amine levels with chronic treatment.

Methods: Male Sprague-Dawley rats (150 gm) were given daily i.p. injections of saline, phenelzine (7.5 or 15 mg/kg), tranylcypromine (5 mg/kg), chlorgyline (0.5 or 1 mg/kg) or pargyline (1 or 4 mg/kg). At 3,7,14,21 and 42 days (6 hours after the last injection) the animals were sacrificed. Brains were rapidly removed and dissected as described by Victor, Baumgarten and Lovenberg (J. Neurochem. 22: 541, 1974). Brain regions were stored in liquid N_2 until assayed, and all samples of a given region were assayed at the same time. TH and TpOH were assayed in annual report Z01 HL 01805-01 HE. Decarboxylase activity was assayed as described in Lovenberg et al. (Lovenberg, W., Weissbach, H., Udenfriend, S.: Aromatic L-amino decarboxylase, J.B.C. 237: 89-93, 1962).

Major Findings: (1) Significant changes from controls (saline) in TH and TpOH activities were seen at 21 and 42 days of phenelzine and tranylcypromine treatment. No changes were observed with pargyline or clorgyline at any time point, or with phenelzine or tranylcypromine treatments less than 21 days. Striatal TH decreased approximately 40% compared to controls, at 21 and 42 days of phenelzine and tranylcypromine. Tegmental TpOH increased significantly (about 20%) compared to controls at 21 and 42 days of phenelzine treatment.

(2) Brainstem 5-hydroxytryptophan (5-HTP) decarboxylase activity was significantly increased with tranylcypromine treatment at all time points. There was no evidence of 5-HTP decarboxylase inhibition by phenelzine.

(3) Clorgyline and pargyline in doses calculated to produce selective inhibition of either the A or B forms of MAO did not produce changes in enzyme activities with chronic treatment.

(4) Kinetic experiments of tegmental TpOH and striatal TH are in progress to establish the nature of the observed changes in enzyme activities at 3 and 6 weeks of treatment.

Significance to Biomedical Research and Institute Program: Since antidepressant drugs require a minimum of several weeks to achieve therapeutic benefit, it is essential to assess the effects of chronic treatment on monoamine levels and synthesis. There are few existing studies of chronic antidepressant effects on amine metabolism in brain.

Both tyrosine hydroxylase and tryptophan hydroxylase are rate-limiting enzymatic steps for the bio-synthesis of dopamine and serotonin, respectively.

With chronic MAO inhibitor treatment the activities of these enzymes were significantly changed in the brain region of their highest activity, i.e., striatum (TH) and mesencephalic tegmentum (TpOH). Lesser but nonsignificant changes were noted for TH in hypothalamus and for TpOH in tectum. The direction of the TH changes is consistent with decreased enzyme synthesis with chronic treatment (3 or more weeks), but changes in enzyme affinity for pteridine cofactor or other kinetic properties are also possible. The apparent increase in TpOH activity with chronic treatment is perplexing and will require additional study. Both the TH and TpOH findings are consistent with two other recent reports in the literature: (Segal, D.S., Kuczenski, R., Mandell, A.J., Theoretical Implications of Drug-Induced Adaptive Regulation for a Biogenic Amine Hypothesis of Affective Disorders. Biol. Psych. 9: 147-159, 1974; Neckers, L.M., Biggio, G., Moja, E., Meek, J.L., Modulation of Brain Tryptophan hydroxylase Activity by Brain Tryptophan Content. JPET 201: 110-116, 1977).

Proposed Course of Project: Kinetic studies of TH, TpOH and 5-HTP decarboxylase will be continued to elucidate the nature of enzymatic changes associated with chronic monoamine oxidase inhibitor treatments.

Publications:

1. Campbell, I.C., Colburn, R., Walker, M.N., Lovenberg, W., and Murphy, D.L.: Norepinephrine and serotonin metabolism in the rat brain: Effects of chronic phenelzine adminstration. Presented at CINP, Quebec City, July, 1976.

2. Skyler, Jay S., Rogol, Alan D., Lovenberg, Walter and Knazek, Richard A.: Characterization of growth hormone and prolactin produced by human pituitary in culture. Endocrinology 100: 283-291, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01865-02 HE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Activation of Tryptophan Hydroxylase: Role of Protein Phosphorylation
(Revised Title)

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

PI:	Donald Kuhn	NIH Postdoctorate 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 20014

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)

Tryptophan hydroxylase activity is enhanced under conditions favorable for phosphorylation of proteins. Preliminary kinetic studies revealed that phosphorylating conditions decreased by half the K_m of tryptophan hydroxylase for its cofactor 6MPH$_4$. A much smaller change ~~in~~ the K_m of tryptophan hydroxylase for its ~~substrate~~ tryptophan was also observed in one experiment. The results of this initial study indicate that the activity of tryptophan hydroxylase like tyrosine hydroxylase, can be affected by cAMP dependent protein kinases.

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Objectives: It has been demonstrated that tyrosine hydroxylase, the rate-limiting enzyme in the synthesis of the catecholamines, dopamine and norepinephrine, can exist in two different activity states corresponding to forms having a high and a low Michaelis constant for the reduced pterin cofactor. Furthermore, the interconversion of tyrosine hydroxylase from one form to the other is apparently mediated by a cAMP dependent protein kinase (Lovenberg et al., PNAS 72:2955-2958, 1975). In an effort to learn more about the control of tryptophan by hydroxylase (TPH), studies similar to those of tyrosine hydroxylase were undertaken to determine if protein phosphorylation might also modulate this enzyme.

Methods: Male Sprague-Dawley rats were decapitated and the mesencephalic tegmentum, which contains essentially all of the 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 (.5 mM), cAMP (.2 mM), and MgAc (10 mM).

Major Findings: Preliminary results indicate that PO_4 conditions reliably decrease by one-half the K_m of tryptophan hydroxylase for the cofactor $6MPH_4$. A similar change in the K_m for tryptophan, though much smaller in magnitude, was also observed in one experiment.

Significance to Biomedical Research and Institute Program: Phosphorylation of a protein (tryptophan hydroxylase) may play a primary role in connecting tryptophan hydroxylase to the low K_m, activated form. This mechanism is potentially very important in the *in vivo* regulation of TPH activity and correspondingly, in 5-HT synthesis. Since TPH activity is not inhibited by its end-product serotonin, it is not likely that changes in the intra-neuronal concentrations of serotonin would alter enzyme activity except under very extreme circumstances. Therefore, the activation of TPH by a protein phosphorylating system is perhaps the single most important mechanism by which TPH responds to increased demands for 5-HT.

Proposed Course of Project: The following studies are planned to more completely assess the effect of phosphorylation of TPH. (1) cGMP will be substituted for cAMP and cAMP, ATP and/or MgAc will be eliminated from various incubations to assess the role of each component in the activation. (2) The effects of PO_4 conditions on TPH from other brain regions will be studied. (3) As a positive control, the activity of tyrosine hydroxylase in the midbrain will also be determined under phosphorylating conditions. (4) Phosphorylating conditions markedly increase the K_i of dopamine for tyrosine hydroxylase. Since catechols are also potent inhibitors of TPH, the possibility that dopamine inhibition of TPH is altered under phosphorylating conditions will also be determined. This mechanism represents an important concept in the regulation of TPH since it is likely that midbrain dopamine levels will be increased in response to phosphorylation of tyrosine hydroxylase. (5) Antibodies directed against a suitable purified TPH will be produced

and the uptake of phosphate (^{32}P) from ATP-γ ^{32}P into the enzyme will be studied by specific immunoprecipitation.

Publications:

1. Lovenberg, W. and Bruckwick, W.: The enzymology of tryptophan hydroxylase. Symposibum on Biochemical and Function of Monoamine Enzymes. Usdin, Earl and Weiner, Norman (Eds.) Marcel Dekker, Inc. In press (1977).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01866-02 HE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Purification 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:	Matthew M. Ames	Research Associate	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 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.2	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 long-term goal of this work is the purification of rat striatal tyrosine hydroxylase. We achieved a significant increase in specific activity via Sepharose 4B chromatography, and have studied hydroxylapatite chromatography. Future studies will utilize the above procedures as well as affinity chromatography and disc gel electrophoresis. We hope to obtain purified preparations for further kinetic characterization.

Objectives: Tyrosine hydroxylase has been partially purified from bovine adrenal tissue by traditional column chromatography techniques. Other methods have included preliminary treatment with trypsin followed by column chromatography methods. Human pheochromocytoma tyrosine hydroxylase has reportedly been purified via salt precipitation, disc gel electrophoresis, and sucrose density gradient treatment. However, attempts at purification of rat striatal enzyme have been much less successful--there are no literature reports of a highly purified preparation. Two of the major problems encountered in purification of striatal tyrosine hydroxylase are the instability of the enzyme and its tendency to aggregate during purification. The latter problem is illustrated by the fact that molecular weight estimates have varied from 35,000-200,000 daltons. The objectives of this study are to improve the purification procedures for isolation of rat striatal tyrosine hydroxylase, and to obtain purified preparations with enough residual activity for kinetic studies.

Methods: Preliminary steps in our purification studies included ammonium sulfate fractionation, equilibrium dialysis, and Sephadex G-25 chromatography. Major purification steps included Sepharose 4B column chromatography in 20 mN potassium phosphate buffer (pH 6.0), hydroxylapatite chromatography with stepwise elution in potassium phosphate buffer (varying concentration gradients), and hydroxylapatite chromatography with continuous elution (gradients from 20 mM to 300-1000 mM). Enzymatic activity was determined by the standard tritium release assay under optimal conditions, and proteins determined by the Lowry method. Visual indication of purity was studied using disc gel electrophoresis under a variety of conditions.

Major Findings: In our previous report we found that while we were able to obtain significant purification, we did not approach purity. The loss of enzyme activity during the purification has until this point been an insurmountable problem in this project and no significant progress has been made in the past year.

Significance to Biomedical Research and Institute Program: Characterization of the enzyme which is rate-determining in the production of catecholamines in the central nervous system would enhance the understanding of the role and regulation of these enzymes in the central nervous system. Purification of tyrosine hydroxylase is a necessary prerequisite to such a characterization and would, therefore, be a significant contribution to biomedical research.

Proposed Course of Project: We plan to continue our present studies with Sepharose 4B and hydroxylapatite chromatography, as well as studying the application of an affinity column to our purification techniques. We also hope to better employ disc gel electrophoresis in interpretation of the purification studies. While we have no specific plans to examine other techniques, we anticipate expanding our methodology in further purification studies.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01867-02 HE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Short Term Regulation of Tyrosine Hydroxylase

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

PI:	Robert C. Rosenberg	Staff Fellow	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI
	Eleanor Bruckwick	Research Assistant	HE NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.4	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 the kinetic and molecular properties of tyrosine hydroxylase (TH) from rat striata in order to elucidate the mechanism of its short term regulation.

Objectives: The objective of this project is to study the mechanisms involved in the short term regulation of tyrosine hydroxylase (TH), as well as the molecular properties of the native enzyme.

Methods: Steady state kinetic techniques are being used to study the properties of tyrosine hydroxylase in crude homogenates of the striatal area of rat brain. Gradient ultracentrifugation and gel filtration are being used to study the molecular properties of native TH.

Major Findings: 1. Kinetics: At subsaturating conditions of substrate tyrosine and natural cofactor, biopterin, TH activity was found to be very sensitive to the concentration of molecular oxygen. The apparent K_m for molecular oxygen under the above condition was found to be 6%. These findings indicate that the oxygen levels in tissue may also play a significant role in regulating TH activity in vivo.

2. Molecular Parameters: The molecular parameters of native tyrosine hydroxylase from rat brain have been determined via independent measurement of the sedimentation coefficient and Stokes radius of the enzyme in the supernatant fraction from crude tissue homogenates. Aggregation of the enzyme, which has prevented this type of measurement in other laboratories has not been observed under the conditions of our experiments. Tyrosine hydroxylase from the caudate nucleus, hypothalamus, and adrenal have been shown to be essentially identical in their gross physical properties and to have a molecular weight of around 216,000.

Significance to Biomedical Research and Institute Program: Tyrosine hydroxylase is the rate limiting enzyme for the synthesis of catecholamines in the central nervous system. Factors which effect the activity of TH can influence the levels of catecholamines. Regulation of TH activity by product or feedback inhibition is well known. It now appears that there are other regulatory modes for this enzyme as well. Studies of these effects can help us elucidate and understand the multivaried response of catecholamine containing neurons to external stress.

Proposed Course of Project: The kinetic experiments with oxygen as the varied substrate will be completed. The effect of trypsin treatment on the molecular properties of TH will be studied.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01868-02 HE

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Regulation of Tyrosine Hydroxylase

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

PI:	Pauline Lerner	Research Chemist	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI
	Peter Nosé	Chemist	HE NHLBI
	Edna K. Gordon	Research Chemist	LCS NIMH

COOPERATING UNITS (if any)

Laboratory of Clinical Science, NIMH

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.4	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 short- and long-term effects of neuroleptic drugs differ both clinically and biochemically. Acute haloperidol treatment causes a kinetic activation of striatal tyrosine hydroxylase. Chronic treatment causes a prompt activation, followed by a delayed, compensatory deactivation below control levels. Tolerance also develops to the stimulating effect of haloperidol on striatal dopamine turnover.

Objectives: Tyrosine hydroxylase is the rate limiting enzyme in the biosynthesis of the catecholamine neurotransmitters. It is known that blockade of central dopaminergic receptors with neuroleptics, such as phenothiazines and butyrophenones, leads to an increase in dopamine turnover. Dopamine synthesis is increased via a rise in the activity of tyrosine hydroxylase. The stimulation of the enzyme is mediated by an increase in the affinity of the tyrosine hydroxylase for its pterin cofactor. We are interested in studying the regulation of tyrosine hydroxylase in rats receiving acute or chronic injections of haloperidol, a butyrophenone type drug. This is of particular interest in light of recent findings which suggest that there may be significant differences between the long-term and short-term effects of neuroleptic treatment on central dopamine metabolism in humans.

Methods: Haloperidol or saline is administered to rats daily by intraperitoneal injection. After appropriate intervals of time, the rats are sacrificed and the striata are removed rapidly. Tyrosine hydroxylase is assayed in striatal extracts by a tritium release method. Dopamine is measured fluorometrically. An index of dopamine turnover is obtained by measuring the dopamine metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) by mass fragmentography.

Major Findings: Our short-term experiments with haloperidol confirm and extend the findings of others. One hour after injection of haloperidol, there is an increase in striatal tyrosine hydroxylase activity. This stimulation is effected by an increase in the affinity of the enzyme for its pterin cofactor. When haloperidol is administered chronically, and tyrosine hydroxylase is measured one hour after the last injection, the usual increase in enzyme activity is seen. However, when the drug is administered chronically and the enzyme is assayed 23 hours after the last injection, the enzyme activity is decreased significantly below control levels. In this case, there is an apparent decrease in the affinity of the enzyme for its pterin cofactor. These changes reveal an adaptive response of tyrosine hydroxylase to chronic neuroleptic treatment.

Acute and chronic haloperidol treatment also have different effects on striatal dopamine turnover. After a single dose of the drug, both DOPAC and HVA are elevated above control levels. However, after long-term drug treatment, DOPAC and HVA are lower in drug-treated rats than in controls. Thus striatal dopamine turnover also develops tolerance to chronic haloperidol treatment.

Significance to Biomedical Research and Institute Program: Although neuroleptic drugs have constituted a major mode of therapy for schizophrenia in the last twenty years, the precise biochemical mechanism by which these drugs exert their antipsychotic effects is not known. The drugs are effective antipsychotic agents only when administered chronically. The biochemical effects of short-term and long-term neuroleptic treatment are quite different. The biochemical responses to acute drug treatment have been extensively investigated. This project is directed towards clarifying the biochemistry of chronic drug treatment.

Proposed Course of Project: We plan to extend our studies on chronic haloperidol treatment to non-striatal dopaminergic sites in the brain.

Publications:

1. Lerner, Pauline, Nosé, Peter, Gordon, Edna K. and Lovenberg, Walter: Haloperidol: Effect of long-term treatment on rat striatal dopamine synthesis and turnover. Science (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01870-01 HE

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Vascular Protein Synthesis in the Spontaneously Hypertensive Rat

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

PI:	W. Lovenberg	Chief, Sec. Biochem. Pharmacol.	HE NHLBI
OTHER:	T. Nakada	Visiting Scientist	HE NHLBI
	Y. Yamori	Visiting Scientist	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertensive-Endocrine Branch

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

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)

Previous studies have shown that the genetic spontaneously hypertensive rats incorporate lysine into non-collagen protein of the mesenteric arteries at a greater rate than do age matched control animals. We have now also observed this phenomenon in the spermatic and testicular arteries. Using these arteries as being typical of small resistance vessels we have examined the effect of antihypertensive agents on this parameter of vascular protein metabolism. In general drugs that prevent or reduce sympathetic nerve input to the tissue tend to normalize the enhanced vascular incorporation of lysine into non-collagen protein in the spontaneously hypertensive rat. These agents include hexamethonium, clonidine, and phenoxybenzamine. Conversely hydralazine which is an effective antihypertensive agent in these animals did not affect this parameter. Of interest is the observation that the β-blocker, propranolol, neither reduced blood pressure or changes amino acid incorporation in these hypertensive rats. Our data are consistent with the concept that the sympathetic nervous system exerts a trophic effect on vascular non-collagen protein synthesis.

Objectives: The purpose of this work was to determine whether sympathetic nerves have a trophic effect on the smooth muscle of the small arteries. The spontaneously hypertensive rats (SHR) which exhibit substantially higher rates of non-collagen protein synthesis in the mesenteric arteries than do their normotensive controls (WKR) were used as the model system. It was, therefore, of interest to determine the effect of sympatholytic agents on blood pressure and amino acid incorporation into non-collagen protein.

Methods: The experiments were begun with age matched groups of SHR, WKR, and a substrain of the SHR designated as stroke-prone. Blood pressures were recorded at the beginning of the experiment and at weekly intervals. The antihypertensive agents were given as follows: hydralazine in drinking water, 80 mg/liter; hexamethonium, 50 mg/kg twice daily sc; clonidine 200 µg/kg twice daily sc; phenoxybenzamine, 1 or 3.5 mg/kg twice daily sc; and propranolol, 3 mg/kg twice daily sc. After 2 weeks of such treatment the animals were fasted overnight and given a pulse of ^{3}H-lysine (0.4 µc/g) and sacrificed after two hours. The small vessels were removed from the animals and the amount of radioactivity incorporated into the non-collagen proteins, collagen, and elastin determined by procedures previously developed in our laboratory.

Major Findings:

(1) The rate of lysine incorporation into non-collagen protein of the spermatic and testicular arteries is 2 to 3 times greater in the SHR and stroke prone SHR than it is in the parent strain (WKR).

(2) The previously observed increase in lysine incorporation in the mesenteric vessel of the hypertensive animals was confirmed.

(3) No changes in lysine incorporation in the heart or aorta were observed.

(4) Incorporation of lysine into collagen and elastin was not different in any of the strains with or without antihypertensive therapy.

(5) Hexamethonium, clonidine and phenoxybenzamine all caused a significant decrease in lysine incorporation into non-collagen protein of the SHR but not of WKR.

(6) Hydralazine and propranolol were without effect on non-collagen synthesis.

(7) Histological studies indicated a good correlation between the increased incorporation of lysine and the development of pathological lesions in the small vessels.

Significance to Biomedical Research and Program of the Institute: The major index of success in the treatment of high blood pressure in man is the simple reduction in the pressure. The current studies using a rat model of hypertension suggest that increased synthesis of muscular protein may be one of the pathogenic factors in the development of the disease. Furthermore, our studies indicate that the vascular hypertrophy may be controlled by the sympathetic nervous system. The treatment with a vasodilator may be very effective in reducing blood pressure, but may not alter the underlying cause of the disease.

Proposed Course of Project: There are several specific experiments planned.

(1) Attempt to measure the incorporation of lysine and/or other amino acids in brain microvessels which will be isolated by a recently described method. If the enhanced amino acid incorporation is observed in this preparation the effects of specific drugs will be examined. We will also study the effect of age on this phenomenon.

(2) We plan to attempt to set up an organ culture system for measuring the incorporation of lysine into non-collagen proteins of the small vessels.

Publications:

Lovenberg, W., Yamabe, H., Nakada, T. and Yamori, Y.: Vascular protein synthesis in the spontaneously hypertensive rat. In: Spontaneous Hypertension: its Pathogenesis and Complications (Proceedings on the Second International Symposium on the Spontaneously Hypertensive Rat), 1977, pp 56-60.

Yamori, Y., Nakada, T., and Lovenberg, W.: Effect of antihypertensive therapy on lysine incorporation into vascular protein of the spontaneously hypertensive rat. European J. Pharmacology 38: 349-355, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01871-01 HE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Characterization of Tyrosine Hydroxylase in NIE-115 Neuroblastoma Cell Cultures

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

PI:	Matthew M. Ames	Research Associate	HE NHLBI
OTHER:	Jeremiah J. Morrissey	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 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.6	

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)

The goal of this work was to characterize tyrosine hydroxylase in the adrenergic NIE-115 neuroblastoma cell system, and to compare the properties of the enzyme with that of rat striatum. Kinetic properties of the enzyme in this cell system were determined and several important differences between the cell enzyme and that from the rat striatum were found and described.

Objectives: Mouse neuroblastoma cell cultures contain the enzymatic machinery necessary for the synthesis and metabolism of neurotransmitters such as acetylcholine, dopamine and norepinephrine. One such cell line, NIE-115, has particularly high levels of tyrosine hydroxylase activity. In conjunction with rat brain studies on the regulation of this enzyme, we felt that certain kinetic experiments using the cell culture system would compliment the work with rat brain preparations. For this purpose, it was first necessary to further characterize tyrosine hydroxylase in the NIE-115 cell line, and compare the results with properties of the rat brain enzyme.

Methods: Cells were grown under standard conditions using Eagles medium containing 10% fetal calf serum. The cells were passaged weekly. Cells were isolated for enzyme analysis by low speed centrifugation, followed by sonication. Tyrosine hydroxylase activity was determined by the tritium release assay method. K_m values were determined by the Lineweaver-Burke method.

Major Findings: It was determined that tyrosine hydroxylase from NIE-115 cells is very sensitive to heat. At 37°, the reaction rate was linear for only 4-5 minutes. In contrast, the rat striatal enzyme reaction rate was linear for at least 20 minutes. Also, more than 90% of the enzymatic activity was associated with the particulate fraction of the cell preparations. In rat brain preparations, greater than 50% of the activity has been found in the soluble fraction. Kinetic studies demonstrated that tyrosine hydroxylase in the NIE-115 cells had a lower apparent K_m for reduced pterin cofactor than the rat brain enzyme. Both enzymes were sensitive to dopamine end-product inhibition. Activation by dibutyrl-cAMP (via an increase in V_{max}) was also demonstrated in the cell enzyme. We also found that tyrosine hydroxylase activity diminished with cell age (passage number), but that this phenonemon was not due to major changes in chromosome number or gross appearance.

Significance to Biomedical Research and Institute Program: Tyrosine hydroxylase is the rate-determining step in the biosynthesis of the catecholamine neurotransmitters. Studies on the mechanism by which this enzyme is regulated are of great importance to the understanding of brain function and the pathology of various disease states, such as hypertension. Certain types of studies can be better performed in a cell system, free of the problems of tissue preparation, distribution or metabolism of drugs, and other confines. For this purpose, an understanding of the properties of tyrosine hydroxylase in such a cell system will be of value for further studies.

Proposed Course of Project: It is anticipated that tyrosine hydroxylase studies in the NIE-115 cell line will be continued, with an emphasis on activation by dibutyl-cAMP. These studies will include the use of agents which would reveal the role of other neural pathways in the regulation of tyrosine hydroxylase in adrenergic neurons.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01872-01 HE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Pteridine Cofactor Activity in Biologic Fluids

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

PI:	Donald S. Robinson	Guest Worker (Univ. Vermont College of Medicine)	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI
	Daniel van Kammen	Chief, Sect. Neuropsychopharmacol.	CNB NIMH
	Robert Post	Chief, Sect. Psychobiol.	BPB NIMH
	Donald Calne	Chief, Exp. Ther. Branch	ETB NINCDS
	Adrian Williams	Clinical Associate	ETB NINCDS

COOPERATING UNITS (if any)
NIMH, Biological Psychiatry Branch; NINCDS, Experimental Therapeutics Branch

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.5	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 investigation of the major determinants of monoamine synthesis and turnover in vivo is of intense scientific interest because monoamine levels in the central nervous system (CNS) play critical roles in neuropsychiatric, neuroendocrine and cardiovascular diseases. Tyrosine hydroxylase is known to be the rate-limiting enzymatic step in the synthesis of dopamine and norepinephrine. Current evidence suggests that in vivo rate of synthesis depends primarily on dopamine concentration (due to end-product inhibition) and pteridine cofactor levels. Thus, pteridine levels in cerebrospinal fluid (CSF) are of interest.

This study was undertaken to detect and monitor CSF pteridine cofactor activity using a phenylalanine hydroxylase assay (Bullard, W.P. et al.,Dynamics and disposition of reduced pterins in rat brain, JPET, in press). Preliminary results show that cofactor activity is detectable in CSF of patients with a variety of neuropsychiatric disorders. Cofactor activity is stable in CSF stored at -70°C in liquid N_2 for a period of several days. The data suggest that patients with neurological disorders which include parkinsonism, multiple sclerosis and Huntington's chorea, have lower mean cofactor activity in CSF than patients with schizophrenia and affective disorders.

Objectives: Since a variety of pathologic diseases may result from defects in monoamine synthesis, it is of interest to assess one possible index of in vivo monoaminergic function, CSF pteridine cofactor activity. Studies of neurologic and psychiatric patients are in progress in collaboration with the Biological Psychiatry Branch, NIMH and the Neurology Service (Dr. Calne). In addition, we are investigating pteridine levels in human CSF using an independent method of assay, crithidia bioassay. Association between CSF cofactor activity and disease states, drug treatments and biologic variables such as age, sex etc. will be investigated.

Methods: Two ml of CSF is freshly collected in an opaque polycarbonate tube, wrapped in aluminum foil to protect it from light, and placed in ice. It is then either assayed within 60 minutes, or stored in liquid N_2 for up to three days prior to assay.

Cerebrospinal fluid (0.3 ml) is transferred to a glass test tube containing 25 µg of sheep liver quinoid dehydropterin reductase (QDPR), 2 mM NADH, ca 1 mg of rat liver phenylalamine hydroxylase (partially purified), 100 µM 4-^{3}H-phenylalanine (specific activity ca 40 mCi/m mol) in a final volume of .430 ml. Following incubation for 90 min. at 30°, the reaction mixture is cooled to 0° in an ice bath, and the reaction arrested by addition of .100 ml of 1.2 M sodium acetate buffer, pH 5.5. To release any tritium in the 3-or 5-position of the tyrosine formed, 25 µl of N-iodosuccinamide (100 ml/ml in DMSO) is added to the cooled samples. After 5 min the released tritium (as ^{3}HOH) is collected by passage of a .450 ml aliquot over a 0.5 x 3 cm Dowex 50-H+ exchange resin. The column is washed twice with 0.65 ml of water, and the total eluent collected in a scintillation vial to which 10 ml of scintiallation cocktail is added. A calibration curve using 6-methyltetrohydropterin (6-MPH_4) standards in bovine serum albumin (4 mg/ml) are run concomitantly. A UV spectrum is obtained on a 50 µM solution of $6MPH_4$ in .01 N HCl just prior to the assay to quantify the concentration of reduced cofactor in the cofactor stock solution. CSF cofactor activity is expressed as p-moles per ml CSF.

Major Findings: (1) Measurable and reasonably stable levels of pteridine-like cofactor activities are detectable in patients over time. Studies are in progress by indepéndent assay methods to determine specific pteridine levels in CSF. (2) Approximately 50 patients with a variety of neuro-psychiatric disorders have been studied to date. Although there is overlap in individual patient values between diagnostic groups, there is a significantly lower mean cofactor activity in neurological patients with basal ganglia disease and multiple sclerosis compared to patients with schizophrenia and affective disorders. (3) CSF cofactor activity seems to be relatively constant in the initial and later aliquots of fluid obtained from a patient. DBH activity is being measured concurrently also to examine for a possible gradient between initial and subsequent aliquots, and also to examine for relationship of DBH to cofactor activity.

Significance to Biomedical Research and Institute Programs: (1) It is important to identify the major determinants of monoamine synthesis and turnover in man, and to assess the relationships of these variables to

biologic variables and disease states. (2) Measurement of CSF cofactor activities in patients should permit assessment of possible deficiencies in disease states where impaired monoamine metabolism has been shown to be an etiologic factor (such as basal ganglia degeneration) as well as in other conditions where altered monoaminergic function has been hypothesized (schizophrenia, affective illness). (3) This project will provide additional insight into mechanisms which control CNS monoamine levels and their relationships to pathologic conditions.

Proposed Course of Project: Assay of patient CSF speciments will be continued in order to enlarge our series of patients with neurologic and psychiatric diseases. In addition, we hope to obtain CSF specimens from patients with other illnesses, including cardiovascular disorders, hypertension etc., and from normal controls.

We also intend to develop more sensitive and specific methods to measure pteridine levels in biologic fluids, such as high pressure liquid chromatography. We hope to expand this project to examine control mechanisms of pteridine synthesis in various tissues.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01873-01 HE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Activation of Brain Tyrosine Hydroxylase by LSD and Other Psychoactive Agents

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

PI:	Donald M. Kuhn	NIH Postdoctorate Fellow	HE NHLBI
OTHER:	Walter Lovenberg	Chief,Sect. Biochem. Pharmacol.	HE NHLBI
	Pauline Lerner	Research Chemist	HE NHLBI
	K. Sankaran	Guest Worker	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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 administration of the potent hallucinogen LSD results in an activation of striatal tyrosine hydroxylase (to 150% of control). The potent neuroleptic haloperidol produces a similar alteration in enzyme activity and when given together the effects of LSD and haloperidol are additive. LSD, like most dopamine receptor antagonists, can activate tyrosine hydroxylase and this activation may be dependent on cAMP.

Objectives: Numerous recent studies have indicated that LSD can act as a mixed agonist/antagonist at dopamine (DA) receptors in brain. LSD does induce an increase in striatal DA synthesis and it apparently increases the activity of a DA-sensitive adenylate cyclase as well. The objective of these experiments is to study the response of the rate-limiting enzyme in catecholamine synthesis, tyrosine hydroxylase, to injections of LSD and other hallucinogenic and/or ergot derivatives. In depth studies will be carried out to determine the mechanisms which mediate LSD-induced changes in the dynamics of central DA biosynthesis.

Methods: Male Sprague-Dawley rats were injected with .5 mg/kg of LSD on ventricle and sacrificed by decapitation 30 or 60 min after injection. The striata were rapidly dissected, frozen, and stored in liquid N_2 until assay. Tyrosine hydroxylase activity was determined by the method reported in Lovenberg et al. (PNAS 72: 2955-2958, 1975).

Major Findings: In preliminary experiments the striatal tyrosine hydroxylase activity in LSD treated rats was increased to 115% of control 30 min. after treatment. By 60 min. this increase was as large as 150%. Haloperidol (.5 mg/kg) activated tyrosine hydroxylase to a similar extent. The effect of LSD plus haloperidol on enzyme activity was greater than either agent given alone.

Significance to Biomedical Research and Institute Program: The DA neuronal system is apparently a target for LSD in brain. On a biochemical level LSD mimics the effects of the major antipsychotic drugs, strangely enough, by increasing DA synthesis. Based on the role the DA system plays in many affective disorders, a better understanding of the mechanisms of action of LSD (with respect to DA) will elucidate how altered DA function contributes to certain brain/behavioral disorders.

Proposed Course of Project: (1) LSD increases cAMP formation in brain slices suggesting an increase in adenylate cyclase activity. However, LSD may also alter the activity of a phosphodiesterase (PDE). Thus, striatal PDE activity will be assayed in the striata of LSD treated rats. (2) The effects of LSD on the kinetics of tyrosine hydroxylase will be determined. (3) Other hallucinogens (e.g., onescaline, dimethyltryptoamine) will be tested for similar effects on tyrosine hydroxylase. (4) Complete time course and dose-response data will be generated. (5) The non-hallucinogenic congeners of LSD, BOL and UML (methysergide) will be tested for effects on striatal tyrosine hydroxylase.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01874-01 HE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Role and Mechanism of Epinephrine Containing Cells in Brain and Adrenal Gland and the regulation of Blood Pressure

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

PI:	Willa B. Phyall	Postdoctorate 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 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.1	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 purpose of this project is to study the relationship between phenylethanolamine N-methyltransferase (PNMT) levels and blood pressure control in hypertensive and normotensive rats. PNMT catalyzes the final step in catecholamine biosynthesis and is both centrally and peripherally located. Recent studies have shown that brain PNMT levels are significantly elevated in hypertensive animals when compared to their controls. Inhibition of this enzyme, particularly in the rich catecholamine containing A_1 and A_2 nuclei of the brainstem, is associated with significant reductions in blood pressure in hypertensive rats. These findings suggest a possible involvement of central noradrenergic neurons in blood pressure regulation as well as a possible involvement of this enzyme in the development of the hypertensive state.

Objectives: Our research goal is to determine if a relationship does in fact exist between PNMT levels and blood pressure control. We are especially concerned with the enzyme in the brain, however, we would like to pursue a course where a comparison could be made between brain and adrenal enzyme activity. This will first require the establishment of developmental and regulatory patterns of the enzyme in normotensive and hypertensive rats. After isolating factors which regulate the enzyme, we would like to see how and if manipulation of these factors affect blood pressure. To determine what effects antihypertensive drug treatment have on enzyme levels is a major part of this project. Finally, we would like to determine the role and mechanism of PNMT inhibition by the identification of basic inhibition mechanisms using pure enzyme preparations.

Methods: Male normotensive and hypertensive rats are being used in these experiments. Animals are treated with various drug regimens for a specified time period. At the end of this period, animals are killed by decapitation. The adrenal glands and whole brains are removed and frozen on dry ice. The brainstem is dissected via a gross method into A_1 and A_2 regions. All tissue is stored in liquid nitrogen until assay time. During drug treatment, blood pressures are recorded in conscious animals by using the tail pressure cuff technique which is an indirect method of measurement. PNMT in brain and adrenal samples are measured by a modification of the method described by Saavedra et al., Nature 248, 1974.

Major Findings: This project is in the early stages of development and at present there are no major findings to report.

Significance to Biomedical Research and Institute Program: Recent evidence sited in the literature as well as results from initial experiments suggests the importance of this project in that it may possibly aid in the elucidation of the mechanism of action of various anti-hypertensive drugs as well as contribute to the awareness of other underlying factors which may be involved in the development of hypertensive disease.

Proposed Course of Project: The following experimental approach will be undertaken in order to determine the role of PNMT in blood pressure control. (1) Examination of enzyme and blood pressure in age matched normotensive and hypertensive untreated animals. (2) Studies on the effects of various pharmacologic agents on enzyme levels and blood pressure in both strains of rats. (3) Studies on the inhibition kinetics of the enzyme.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01875-01 HE

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
The Effect of Vasodepressor Drugs on Biogenic Amine Turnover in the CNS.

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

PI:	T. Kent Keeton	Research Associate	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 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	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)

The ultimate goal of this project is to determine whether beta-adrenergic blocking drugs lower blood pressure by an action in the central nervous system that includes alteration of noradrenergic nerve activity. We have determined that propranolol causes a selective increase in norepinephrine turnover in the corpus striatum, cerebral cortex and cerebellum with no changes occurring in the medulla, midbrain-hippocampus or hypothalamus. Other beta-blockers such as timolol and butoxamine cause a decrease in whole brain norepinephrine turnover. In future studies we plan to determine the effect of a large number of beta-blocking drugs on norepinephrine release in six discrete brain regions and attempt to correlate these changes with the antihypertensive effect of these drugs.

Objectives: We hope to determine if beta-adrenergic blocking drugs lower blood pressure by an action in the central nervous system that involves changes in noradrenergic nerve activity. By comparing the central biochemical effects of beta-blockers with the effects of anti-hypertensive drugs that are known to decrease blood pressure by an action in the CNS (e.g., clonidine), we hope to determine if a single common denominator exists.

Methods: Norepinephrine turnover is determined indirectly by measuring the concentration of 3-methoxy-4-hydroxyphenylglycol sulfate (MOPEG-SO_4) in brain tissue after treatment of the animals with different anti-hypertensive agents. This compound is the major metabolite of norepinephrine in the CNS and has been demonstrated to be a good index of noradrenergic nerve transsmission in the CNS. MOPEG-SO_4 is assayed by fluorescent spectrophotometry after isolation on a DEAE-sephadex A-25 column. Rat brains are dissected into the cerebral cortex, hypothalamus, corpus striatum, medulla cerebellum, and midbrain-hippocampus prior to assay of MOPEG-SO_4. Blood pressure is measured in conscious rats via chronic aortic cannulae or by the indirect tail-cuff method.

Major Findings: Neither acute, sub-acute, or chronic treatment with propranolol caused any change in the whole brain concentration of MOPEG-SO_4. Acute propranolol did cause a selective increase in MOPEG-SO_4 concentration in the cerebral cortex, corpus striatum, and cerebellum with no change occurring in the medulla, hypothalamus, or midbrain-hippocampus. By comparison, the alpha-adrenergic blocking drug phenoxybenzamine increased norepinephrine turnover in all brain regions whereas the alpha agonist clonidine caused a non-specific decrement in MOPEG-SO_4 concentration in all brain regions. The beta-blockers timolol and butoxamine decreased, while pindolol increased, norepinephrine turnover in whole brains of normal Kyoto-Wistar and Okamoto strain spontaneous hypertensive rats. In addition, the combined alpha-and beta-adrenergic blocking drug labetalol had no effect on MOPEG-SO_4 concentration in normal Wistar rats whereas the vasodilatory anti-hypertensive drug prazosin caused a large increase in norepinephrine turnover in all six brain areas.

Significance to Biomedical Research and Institute Program: All beta-adrenergic blocking drugs tested thus far in hypertensive humans have been found to lower blood pressure. However, the exact mechanism by which these drugs lower blood pressure has not been elucidated. Many clinical researchers believe that these drugs act within the CNS to decrease efferent sympathetic discharge to peripheral organs, e.g., blood vessels. Because noradrenergic fiber tracts within the CNS play a critical role in controlling cardiovascular function, it is important to know the effect of beta-blockers on the functional state of these tracts. This insight may help to determine how beta-adrenergic blocking drugs lower blood pressure and how they precipitate certain untoward effects.

Proposed Course of Project: We plan to continue our present studies with beta-adrenergic blocking drugs and norepinephrine turnover. After determination of the neurochemical changes elicited by these agents, blood pressure correlates will be determined for the appropriate doses and time periods.

ANNUAL REPORT OF THE
MOLECULAR DISEASE BRANCH
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
July 1, 1976 through September 30, 1977

The overall objective of the research program of the Molecular Disease Branch is to elucidate the molecular mechanism involved in plasma lipoprotein biosynthesis, transport,and degradation in normal individuals and patients with disorders of lipid metabolism and atherosclerosis.

Over the last few years it has become increasingly apparent that the routinely employed classification systems of plasma lipoproteins based on electrophoresis and density fractionation are inadequate. The polydispersity of plasma lipoproteins with a given density fraction has been clearly documented. It has now become necessary to develop new concepts and laboratory techniques within this framework for future research in the field of lipoprotein metabolism. Our recent efforts have been directed toward the formulation of a concept of plasma lipoproteins which encompasses the current available knowledge of lipoprotein structure and function (for detailed review see Osborne, J.C. Jr. and Brewer, H.B. Jr.: Adv. in Prot. Chem. 31:253-337, 1977). The rapid advances which have occurred in recent years in the lipoprotein field have resulted in a lipoprotein nomenclature which is imprecise and often ambiguous. A more rigorous attention to general terminology is now required in order to achieve a nomenclature which is specific and encompasses the information available in the field. We have thus adopted the following three specific definitions: an apolipoprotein is a homogeneous protein, composed of a single polypeptide chain or several polypeptide chains held together by covalent bonds, containing no detectable non-covalently bound lipid, which associates with or forms an integral part of a lipoprotein and/or lipoprotein particle in plasma; a lipoprotein is an apolipoprotein-lipid complex held together by non-covalent bonds in which the lipid moieties are arranged in an anisotrophic manner such that there are no clearly defined surface areas where polar head groups shield an intramolecular hydrophobic region from solvent; a lipoprotein particle is an apolipoprotein-lipid complex held together by non-covalent bonds in which there are extensive regions where the polar head groups of lipids form a surface area that shields an intramolecular hydrophobic region from solvent; a plasma lipoprotein is a general term used when a clear distinction between the above categories is not intended. Categorization of the different plasma lipoproteins is best achieved by delineation of their specific apolipoprotein content. The composition of the plasma lipoproteins at any moment in time is governed entirely by the laws of mass action, with the specific composition of each complex dependent on the relative affinity of each component for each complex in the system. Within this context the concentration of, for example, apolipoprotein C-II attached to triglyceride rich particles within VLDL, or phospholipid-cholesterol rich particles in HDL is dependent on the concentration of the respective particles and the relatively affinity of C-II for each class of particles. The association of lipoproteins and lipoprotein particles is also governed by the laws of mass action. The association of lipoprotein particle containing the B protein (LpB) and a lipoprotein-lipoprotein particle containing the E protein (LpE) to form a LpB, E complex will depend on the concentration and relative affinity of the individual components. "Primary" plasma lipoproteins (LpB, LpE) have been defined as plasma lipoproteins which reversibly associate with other lipoproteins

or lipoprotein particles forming "secondary" plasma lipoproteins (LpB, E). Due to the laws of mass action, it may be difficult to isolate "secondary" plasma lipoproteins from plasma due to dissociation. The extent of dissociation of "secondary" complexes upon fractionation is critically dependent upon the affinity of the "primary" components for one another, and the specific isolation procedure(s) employed. For instance, if the forces involved in the formation of a "secondary" particle are primarily electrostatic, an isolation procedure in the presence of high salt concentration, as occurs during fractionation by ultracentrifugation, would lead to dissociation of all or part of the "secondary" complex. An example of this dissociation is the loss of A-I and E from "secondary" particles during preparative ultracentrifugation. This conceptualization of plasma lipoprotein as a polydisperse collection of lipoproteins-lipoprotein particles, the composition of each being governed by the laws of mass action, should facilitate the design and interpretation of research in the field of lipoprotein structure and metabolism.

Current research in the Molecular Disease Branch involves the systematic investigation of the enzymatic regulation of cholesterol biosynthesis, chemistry and metabolism of plasma apolipoproteins and lipoproteins, apolipoproteins which function as structural components of plasma lipoproteins and cofactors for the enzymatic degradation of the constitutive lipids.

A detailed analysis of the enzymatic regulation of cholesterol biosynthesis has been continued. The rate limiting enzyme, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA reductase) has been purified to electrophoretic homogenity by affinity chromatography from chicken liver. The purified enzyme is a single band on disc gel electrophoresis (PAGE), SDS PAGE, and PAGE in 6M urea. The monomer molecular weight is 18,000 by SDS PAGE which is significantly lower than the 50-60,000 molecular weight reported for rat HMG-CoA reductase. Coenzyme A, acyl CoA esters, and 3-hydroxy-3-methylglutaric acid (HMG) were shown to inhibit HMG-CoA reductase activity. Purified reductase was also inhibited by ATP-Mg^{++} suggesting that phosphorylation of the enzyme was associated with loss of enzymatic activity. Treatment of the inactivated enzyme with a cytosolic fraction rich in phosphotase restored enzymatic activity. These results are consistent with the concept that phosphorylation-dephosphorylation may be an important cellular mechanism for control of enzyme activity. A monospecific antibody to chicken HMG-CoA reductase was developed in goats. This antibody will be used to establish an electroimmunoassay for the quantitation of reductase. A method for quantitation of HMG-CoA reductase will permit a detailed analysis of the regulatory mechanisms involved in the control of enzyme concentration versus enzymatic activity under a number of different physiological conditions.

The regulation of HMG-CoA reductase activity in tissue culture fibroblasts from normal subjects, and patients with disorders of lipid metabolism continue to be investigated. The effect of specific lipoprotein particles (LpB, LpE, LpD, and LpA-I, A-II) have been prepared in collaboration with Dr. Pierre Alaupovic (Oklahoma City, OK) and are being evaluated in tissue culture. Recent analysis of LpB and LpB,E particles have indicated that both particles are able to bind to fibroblasts and inhibit HMG-CoA reductase. These results are consistent with the view that apoE is not necessary for the interaction of lipoprotein particles with fibroblasts to regulate reductase activity. Previous studies by our laboratory and other investigators have shown that

lipoprotein particles containing Lpa without B can also regulate HMG-CoA reductase in cultured fibroblasts.

Prerequisite to our understanding of the molecular mechanisms involved in lipid-protein interactions of plasma lipoproteins in a detailed knowledge of the chemistry and structure of the apolipoproteins. During the last several years, extensive investigation has focused on the isolation, characterization, and structural analysis of the human apolipoproteins. The amino acid sequences of A-II, C-III, and CI have been completed in the Molecular Disease Branch. During the last year the complete amino acid sequence of human A-I has been determined. A-I is a 243 amino acid single chain protein containing no covalently bound carbohydrate or disulfide bridges. The sequence of A-I is similar to the structures of A-II, C-III, and C-I in containing no long segments of hydrophobic residues. The lipid binding properties of apolipoproteins appear to be dependent on the secondary, tertiary, and quaternary structure of the protein. Regions of the amino acid sequence of all apolipoproteins, thus far examined, can be modeled into amphipathic helices with one surface hydrophobic and the other hydrophilic. These amphipathic helices have been proposed to be important in both protein-protein and protein-lipid interactions. Apolipoproteins D and E have been isolated and are being extensively characterized with regard to their amino acid composition and structural properties. ApoE is of particular interest in lipoprotein metabolism as it is elevated in familial dysbetalipoproteinemia and in animals fed diets high in cholesterol. ApoD has recently been proposed as a cofactor for lecithin cholesterol acyltransferase (LCAT) and may be important in the transport and esterification of plasma cholesterol. Investigation continues on the comparison of the composition and sequence of the A-II and C apolipoproteins from normal individuals and dyslipoproteinemic patients.

Of major importance over the last two years has been the recognition that plasma apolipoproteins undergo reversible self-association. Of particular note has been the observation that a major increase in organized structure occurs with self-association. The molecular properties of apolipoproteins have now been shown to be quite sensitive to several experimental conditions, including protein concentration, solvent composition, pH, temperature, and pressure. During the last year a detailed analysis of C-I revealed that this apolipoprotein also self-associated with a monomer-dimer-trimer association scheme. On association, the structure increased from essentially a randon coil in the monomer to a globular protein in the associated species. The increase in structure of C-I with association is greater than any protein previously reported in the literature. The molecular properties of C-III have been investigated and found to be significantly influenced by carbohydrate content. It is inferred from these data that one of the important factors in the molecular interaction and metabolism of plasma lipoproteins and lipoprotein particles is the specific conformation of the apolipoproteins. These experimental observations are of fundamental importance in the analysis of the molecular mechanisms involved in association of apolipoproteins to lipoproteins-lipoprotein particles, and interaction of different lipoproteins-lipoprotein particles.

The quantitation of plasma apolipoproteins is necessary for the compositional analysis of lipoproteins, lipoprotein particles, and plasma density classes. In collaboration with Dr. Pierre Alaupovic, antibodies to all 8 major apolipoproteins have been developed and electroimmunoassays developed for each apolipoprotein. Compositional analysis of 6 patients with Tangier

disease have revealed marked heterogenity of the concentration of the apolipoproteins. These results are consistent with the possibility that different kindreds of patients with Tangier disease may have different metabolic defects.

Lipoprotein particles with defined apolipoprotein composition are being isolated and characterized from human plasma. Using immunological techniques we have been able to confirm the presence of several discrete lipoprotein particles in unfractionated plasma. The isolated lipoprotein particles (ex. LpB,E; LpB; LpA-I, A-II) have been studied in tissue culture with regard to the regulation of HMG-CoA reductase. The association and metabolism of several of these isolated particles will be studied during the next year.

Investigation of the clinical disorders of lipoprotein metabolism continue to be an area of fruitful research. Of particular interest in the field of lipoprotein research has been the study of plasma lipoproteins within HDL. HDL cholesterol has been recently extensively analyzed in respective epidemiological studies and noted to be a negative risk factor for atherosclerosis. To facilitate the clinical determination of cholesterol in density fractions of plasma and to expedite the categorization of patients into phenotypes, we have developed a new micromethod for the rapid, reliable fractionation of plasma lipoproteins, and quantitation of their cholesterol content. This method requires 350 µliters of plasma and can be completed in 3 hours. A prototype enzymatic-oxygen electrode cholesterol analyzer, table top air driven ultracentrifuge, and centrifuge tube aspirator were developed by Beckman Instruments, California, in collaboration with the Molecular Disease Branch Staff. Excellent correlation coefficients were obtained when the enzyme electrode analyzer was compared with the Technicon Autoanalyzer II and Smac systems. Cholesterol content of fractionated plasma lipoproteins (VLDL, LDL and HDL) determined by the new micromethod was in excellent agreement with the values obtained by the procedures standardized by the Lipid Research Clinics. This new method should facilitate the measurement of cholesterol in lipoprotein fractions by the routine clinical laboratory.

Studies of the metabolism of plasma lipoproteins have been continued during the last year. Of particular relevance have been the metabolic studies on plasma lipoproteins within HDL. Estrogen administration was associated with an increase in plasma VLDL and HDL, mainly HDL_2. Plasma lipoprotein elevations were due principally to an increase in lipid (70-80 percent), rather than protein (10-15 percent). Plasma lipoprotein elevations appear to be largely due to increased synthesis, rather than decreased catabolism.

Metabolism of the plasma lipoproteins has previously been studied by radiolabeling of plasma density fractions. To facilitate the study of the metabolism of apolipoproteins associated with specific lipoproteins-lipoprotein particles we have analyzed the kinetics of radiolabeled A-I and A-II and compared it to A-I and A-II radiolabeled in HDL. Radiolabeled A-I and A-II rapidly associated with lipoprotein particles within HDL, and were catabolized at similar rates as A-I and A-II in radiolabeled HDL_{1-3}. In addition, simultaneous studies with ^{125}I HDL_2 (1.063-1.125 g/ml) and ^{131}I HDL_3 (1.125-1.21 g/ml) demonstrated rapid exchange of radiolabeled A-I and A-II, and a similar catabolic rate for both density fractions. These results are consistent

with the view that the affinity of A-I and A-II for lipoprotein particles within the subfractions of HDL are nearly equal. Radiolabeled A-I and A-II, therefore, rapidly exchange between the individual lipoprotein particles. The catabolism of radiolabeled A-I and A-II were enhanced in patients with hyperchylomicronemia (Type I phenotype), and markedly increased in Tangier patients. These latter results are consistent with the catabolic defect in A metabolism proposed in last year's annual report. The ability to study apolipoprotein metabolism by radiolabeling the isolated apolipoprotein and reassociating the apolipoprotein with plasma lipoprotein particles hearlds a new era in the study of lipoprotein metabolism. With these techniques, the metabolism of several apolipoproteins will be able to be studied simultaneously in a single study.

Simultaneous ^{125}VLDL and ^{131}I LDL studies in patients with homozygous familial hypercholesterolemia have been completed and indicate that these patients have increased LDL (apoB) systhesis as well as defective catabolism. In addition, a significant fraction of plasma apoB enters the circulation in lipoprotein particles with a hydrated density of IDL or LDL.

The clinical treatment of patients with familial homozygous hypercholesterolemia has been relatively unsatisfactory. Compensatory increases in synthesis of apoB may contribute to the poor response observed with drugs designed to increase apoB catabolism. A therapeutic trial has been initiated with HMG, a known inhibitor of HMG-CoA reductase and cholesterol biosynthesis. This agent alone, or in combination with drugs which increase Apo B catabolism (ex. cholestyramine), may be effective in normalizing plasma cholesterol in these patients.

The Type II Coronary Intervention Study, designed to test the hypothesis that reducing plasma LDL cholesterol levels will favorably affect the progress of established coronary artery disease, is now entering its sixth year. Results thus far have demonstrated that patients in the program are dying or have myocardial infarctions at a much lower rate than groups at other institutions or in the cadre of patients at NIH with similar degrees of coronary disease who normalize on diet and were discharged from the program. A systematic investigation of the results obtained by angiography have established that this technique will be able to be used to evaluate the progression of coronary artery disease.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02002-03 MDB

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
NHLBI Type II Coronary Intervention Study

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

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Other:	Robert L. Levy, M.D.	Director		NHLBI
	Stephen E. Epstein, M.D.	Chief	CB	NHLBI
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	James Battaglini, M.D.		MDB	NHLBI
	Beverly Rogers, R.N.	Registered Nurse	MDB	NHLBI
	Marjorie Myrianthopoulos	Dietitian	MDB	NHLBI

COOPERATING UNITS (if any) Cardiology Branch, NHLBI; Lipid Metabolism, NHLBI; Radiology Department, NIH; Pulmonary Branch, NHLBI; Clinical Center Administration, NIH; Data Management Branch, Division of Computer Research and Technology, NIH

LAB/BRANCH
Molecular Disease Branch

SE
Section on Lipoproteins

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
8	5.5	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)

This study may answer or shed considerable light on four questions of great interest to the Institute and to all those involved in heart research.

1. Is angiography a reliable and useful tool for the evaluation of coronary artery disease? Can changes on angiography be read reliably enough to serve as an endpoint for scientific studies or important clinical decisions?

2. Can we significantly decrease the incidence of M.I. or death without any additional new treatments or medications?

3. Will lowering cholesterol slow down, stop, or reverse the progression of coronary artery disease?

4. Which populations of Type II patients would appear to do well on standard therapy and which do poorly.

Project Description

Objective:

The primary aim of this study was to determine whether lowering the LDL cholesterol in patients with premature coronary artery disease and Type II will slow, stop, or reverse the progression of coronary artery disease (i.e., to test the lipid hypothesis in a specific group of high risk patients).

Methods Employed:

Two hundred-fifty patients with coronary artery disease demonstrated by coronary angiography, who met all the criteria outlined in the protocol, were to be randomly split into two equal groups (treatment--24 grams cholestyramine and diet control--control--24 grams placebo and diet control). They would be followed in a double blind fashion monthly for two to five years. Repeat coronary angiography was to be performed at two years and at five years.

The end points were to be: (1) a significant difference in the progression of coronary disease as shown on angiography, or (2) a significant difference in new myocardial infarctions or death, or (3) lack of any of the above at five years.

Because (1) the rate of recruitment has been so slow, (2) the patients have done much better than expected, (3) the data analysis of the information obtained has been very difficult, and (4) the reliability of the endpoint (angiography) has been questioned, it was decided to stop recruitment and to evaluate the reliability of the endpoint with a separate study. This study has been done and shows the endpoint can be reliable if certain conditions are met. Therefore, continued in-depth studies on the current patients including repeat angiography at a 5-5½ year time interval are being performed to evaluate why they are doing so well.

Major Findings:

1. Patients in the program are dying and having heart attacks at a much lower rate than outside groups, even after correcting for severity of coronary disease on angiography, ventricular disease, age and sex.

2. Patients in the program are dying and having heart attacks at a much lower rate than patients with similar indications of coronary disease who normalized on the diet and were discharged from the program for this reason.

3. The reliability of the angiogram as a scientific tool for measuring progression of coronary disease has been established.

4. Our results show:

   a) much less progression of CAD in study patients at two years than any other patient group reported upon

b) regression in some patients (10%)--previously unreported except 2-3 individual cases mentioned with other papers

5. Evaluation of our HDL data confirms its independence as a risk factor in our population.

6. An additional year's experience has confirmed all of the preliminary observations made on previous yearly reports and negated none of them.

Significance to Biomedical Research and the Program of the Institute:

This study may answer or shed considerable light on three questions of great interest to the Institute and to all those involved in heart research.

1. Can we significantly decrease the incidence of M.I. or death without any additional new treatments or medications?

2. Will lowering cholesterol slow down, stop or reverse the progression of coronary artery disease?

3. Are subpopulations who will do well and/or poorly on standard therapy definable?

Proposed Course:

Follow-up of already randomized subjects will continue including 5-year catheterizations. Data analysis in areas concerning 1) coronary calcifications as an indication of CAD, 2) HDL as an independent risk factor, 3) reproducibility of angiographic readings, 4) natural history of our population, 5) determinants of a false (+) exercise test and others are proceeding. We hope to develop a working relationship with a strong outside computer group within the next year, and most of our efforts will be in this direction.

Publications:

1. Belmaker, R. H., Pollin, W., Jenkins, C. D., and Brensike, J. F.: Coronary prone behavior pattern in a sample of Type II hypercholesteremic patients. J. Psychosomatic Research 20: 591-594, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02003-06 MDB

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
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

PI:	Ernst J. Schaefer, M.D.	Staff Associate	MDB	NHLBI
Other:	H. Bryan Brewer, Jr., M.D.	Chief	MDB	NHLBI
	Leslie L. Jenkins, M.S.	Biologist	MDB	NHLBI

COOPERATING UNITS (if any) Loren Zech, M.D., Laboratory of Theoretical Biology, NCI; David Foster, Ph.D., Laboratory of Theoretical Biology, NCI; Mones Berman, Ph.D., Laboratory of Theoretical Biology, NCI; Frank T. Lindgren, Ph.D., Univ. of California, Berkeley, CA

LAB/BRANCH
Molecular Disease Branch

St
Peptide Chemistry Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	1.5	2.0

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☒ (a1) MINORS ☒ (a2) INTERVIEWS

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

Studies elucidating the behavior of $\underline{HDL_2}$ and $\underline{HDL_3}$ indicate that the A apoproteins of the subfractions of HDL are in rapid equilibrium, and are catabolized at a similar rate in the plasma. HDL apoprotein metabolism studies show that apoprotein A-I and apoprotein A-II readily associate with HDL when injected into normal subjects and subsequently are catabolized at the same rate as HDL. Apoprotein A-I and A-II studies perform in hyperchylomicronemic subjects and patients with Tangier disease, demonstrated increased catabolism.

Investigations into the effect of estrogens on plasma lipoproteins indicate that estrogen administration causes increases in VLDL and HDL_2 due to increased synthesis of these lipoproteins. Simultaneous ^{125}I VLDL and ^{131}I LDL studies in patients with homozygous familial hypercholesterolemia (FH) show that these patients have both increased production as well as defective catabolism of LDL B protein. In addition, a significant fraction of apoB appears to enter the circulation with either IDL or LDL.

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Project Description:

Objectives:

1) To complete work on apoA-I and apoA-II metabolism in normal and dyslipoproteinemic subjects.

2) To further elucidate the relationship between chylomicron apoprotein and HDL apoprotein (apoA-I and apoA-II) metabolism in normal and dyslipoproteinemic subjects (including patients with type I hyperlipoproteinemia and Tangier disease).

3) To study the effect of 3-hydroxy-3-methylglutaric acid (HMG) on lipoprotein metabolism in patients with familial hypercholesterolemia.

4) To begin investigations on the metabolisms of the C apoprotein (apoC-I, apoC-II, and apoC-III).

Methods Employed:

Lipoprotein isolation methodology as well as gel electrophoretic techniques have been described in previous annual reports. The use of two isotopes, ^{125}I and ^{131}I, to separately label lipoprotein fractions or apoproteins has significantly increased the quantity of data which can be derived from individual metabolic studies. Techniques have been developed for iodinating apoA-I and apoA-II using a modification of the iodine monochloride method. This methodology will be extended to other apoproteins during the next year. Other members of the branch are currently establishing methodology for plasma apoprotein measurements with electroimmunoassys which will facilitate future metabolic studies.

Major Findings:

In the last twelve months, forty-two individual lipoprotein studies were completed in fifteen subjects. Simultaneous ^{125}I VLDL and ^{131}I LDL studies were performed in four patients with homozygous familial hypercholesterolemia and in one normal subject before and during estrogen administration. In another normal volunteer, ^{125}I VLDL and ^{131}I HDL_2 were analyzed before and during estrogen administration. Simultaneous ^{125}I HDL_2 and ^{131}I HDL_3 studies were also performed in two normals, completing the studies on HDL subfractions.

Chylomicron apoprotein metabolism was examined utilizing chylomicrons isolated from a patient with a chylous effusion. The chylomicrons were isolated from the effusion, labeled, and reinfusioned into the patient. Chylomicron apoprotein metabolism was also studied in one Tangier patient, two normals and two patients with Type I hyperlipoproteinemia, utilizing chylomicrons isolated from plasma.

Kinetic analysis of ^{125}I apoA-I and ^{131}I apoA-II has been carried out in 4 normals, 2 Type I patients, and one Tangier patient, and compared with HDL metabolic studies in the same patients.

1. Estrogen administration to five normal females caused a rise in plasma VLDL and HDL, mainly HDL_2. The plasma lipoprotein elevations appear to be largely due to increased synthesis rather than decreased catabolism.

2. Simultaneous ^{125}I HDL_2 (1.063-1.125 g/ml) and ^{131}I HDL_3 (1.125-1.21 g/ml) studies in three normals indicate that the protein moiety of the two HDL subfractions are in equilibrium, and have the same catabolic rate in plasma.

3. Simultaneous ^{125}I VLDL and ^{131}I LDL studies in four patients with homozygous familial hypercholesterolemia indicate that these patients have increased LDL-apoB synthesis as well as defective catabolism, and that a significant quantity of apoB enters the circulation with IDL or LDL.

4. Kinetic analysis of radiolabeled apoA-I and apoA-II demonstrated that both proteins are catabolized at rates similar to HDL in plasma. A apoprotein catabolism was enhanced in hyperchylomicronemic patients and markedly increased in patients with Tangier Disease.

5. Chylomicron apoprotein studies are interpreted as indicating that chylomicron apoproteins may be the precursor for HDL apoproteins, (namely apoA-I and apoA-II).

Significance to Biomedical Research and the Program of the Institute:

High levels of LDL and low levels of HDL have been shown to be independent risk factors for the development of coronary artery disease, the leading cause of death in the U.S. population. The findings described above provide insights into factors regulating the levels of these lipoproteins.

Proposed Course:

Continuing objectives include further study of lipoprotein and apoprotein metabolism in normal and dyslipoproteinemic subjects. Future work will focus on further elucidation of A apoprotein metabolism, as well as the metabolism of apoB and the individual C apoproteins.

Publications:

1. Schaefer, E. J., Levy, R. I., Jenkins, L. L., Brewer, H. B., Jr.: The effects of estrogen administration on human lipoprotein metabolism. Sixth International Symposium on Drugs Affecting Lipid Metabolism, Philadelphia, 1977, in press.

2. Phair, R. D., Hall, M., Bilheimer, D. W., Levy, R. I, Goebel, R. H., and Berman, M.: Modeling lipoprotein metabolism in man. In Proceding of the 1976 Summer Computer Simulation Conference. Simulation Councils Inc., La Jolla, 1976, pp. 486-492.

3. Berman, M., Hall, M. III, Levy, R. I., Eisenberg, S., Bilheimer, D.W., Phair, R. D., and Goebel, R. H.: Metabolism of apoB and apoC lipoproteins in man: kinetic studies in normals and in hyperlipoproteinemics. J. Lipid Research, in press.

4. Blum, C. B, Levy, R. I., Eisenberg, S., Hall, M. III, Goebel, R. H., and Berman, M.: High density lipoprotein metabolism in man. J. Clinical Invest., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02010-06 MDB

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Structure and Function of the Plasma Lipoproteins

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

PI:	H. Bryan Brewer, Jr., M.D.	Chief	MDB	NHLBI
Other:	Fairwell Thomas, Ph.D.	Visiting Scientist	MDB	NHLBI
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	Rosemary Ronan, B.A.	Chemist	MDB	NHLBI
	Ann LaRue, B.A.	Chemist	MDB	NHLBI
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John Gwynne, M.D., Univ. of North Carolina, Chapel Hill, N.C.

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Section on Peptide Chemistry

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NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The complete amino acid sequence of human A-I has been determined. A-I is a 243 amino acid single chain protein containing no carbohydrate or disulfide bridges. A-I contains no long segments of hydrophobic residues, and the lipid binding properties are related to the secondary, tertiary and quaternary structure of the proteins.

ApoD, an apolipoprotein recently proposed as a cofactor for lecithin cholesterol acyl transferase, has been isolated and characterized. Purified apoD is a single band on SDS electrophoresis, and the amino acid composition and immunological properties have been determined. A new large scale sequencer cup for automated Edman degradations has been developed. With this new method, the length of degradation on a single sequencer run has been extended 2 fold, and the ability to sequence large proteins (>250 residues) has been significantly improved.

Project Description

1) Objective:

Determination of the complete amino acid sequence of human A-I.

Methods Employed:

The methods of sequence analysis of human A-I have been detailed in previous annual reports. High density lipoproteins (HDL) were isolated by preparative ultracentrifugation (density 1.063 to 1.2 g/ml), and delipidated with chloroform-methanol (2:1, v/v). A-I was isolated from delipidated HDL by gel filtration on Sephadex G-200 (superfine) in 8M urea. A-I was cleaved into peptide fragments for sequence analysis by cyanogen bromide, trypsin, citroconylation followed by trypsin, hydroxylamine, skatole, cyclohexandione, and staph aureus protease. Peptides were purified to homogenity by gel permeation and ion exchange chromatography. Isolated peptides were assayed for purity by amino acid analysis, thin layer chromatography, disc gel electrophoresis, and amino terminal analysis. The sequence of intact A-I and peptide fragments was determined by manual and automated Edman-phenylisothiocyanate degradation. Phenylthiohydantoin amino acids (PTH amino acids) were identified by gas-liquid and high speed liquid chromatography and mass spectroscopy.

Major findings:

The complete amino acid sequence of human A-I was determined by manual and automated degradation of intact and peptide fragments of A-I. A-I is a single chain protein composed of 243 amino acids. The complete amino acid sequence is as follows: Asp_1-Glu-Pro-Pro-Gln-Ser-Pro-Trp-Asp-Arg-Val-Lys-Asp-Leu-Ala-Thr-Val-Tyr-Val-Asp-Val-Leu-Lys-Asp-Ser_{25}-Gly-Arg-Asp-Tyr-Val-Ser-Gln-Phe-Gly-Gly-Ser-Ala-Leu-Gly-Lys-Gln-Leu-Asn-Leu-Lys-Leu-Leu-Asp-Asn-Trp_{50}-Asp-Ser-Val-Thr-Ser-Thr-Phe-Ser-Lys-Leu-Arg-Glu-Gln-Leu-Gly-Pro-Val-Thr-Gln-Glu-Phe-Trp-Asp-Asn-Leu_{75}-Glu-Lys-Glu-Thr-Glu-Gly-Leu-Arg-Gln-Glu-Met-Ser-Lys-Asp-Leu-Glu-Glu-Val-Lys-Ala-Lys-Val-Gln-Pro-Tyr_{100}-Leu-Asp-Asp-Phe-Gln-Lys-Lys-Trp-Gln-Gln-Gln-Met-Glu-Leu-Tyr-Arg-Gln-Lys-Val-Glu-Pro-Leu-Arg-Ala-Glu_{125}-Leu-Gln-Glu-Gly-Ala-Arg-Gln-Lys-Leu-His-Glu-Leu-Gln-Glu-Lys-Leu-Ser-Pro-Leu-Gly-Gln-Gln-Met-Arg-Asp_{150}-Arg-Ala-Arg-Ala-His-Val-Asp-Ala-Leu-Arg-Thr-His-Leu-Ala-Pro-Tyr-Ser-Asp-Glu-Leu-Arg-Gln- Arg-Leu-Ala_{175}- Ala-Arg-Leu-Glu-Ala-Leu-Lys-Glu-Asn-Gly -Gly-Ala-Arg-Leu-Ala-Glu-Tyr-His-Ala-Lys-Ala-Thr-Thr-His-Leu_{200}-Ser-Thr-Leu-Ser-Glu-Lys-Ala-Lys-Pro-Ala-Leu-Glu-Asp-Leu-Arg-Gln-Gly-Leu-Leu-Pro-Val-Leu-Glu-Ser-Phe_{225}-Lys-Val-Ser-Phe-Leu-Ser-Ala-Leu-Glu-Glu-Tyr-Thr-Lys-Lys-Leu-Asn-Thr-Gln_{243}.

The sequence of human A-I determined in our laboratory is at variance with the published sequence (Baker, et al, Proc. Natl Acad. Sci.: 71, 3631, 1974). In our studies A-I is 243 vs 245 amino acid in length, and 25 of the 243 residues are different including the insertion of amino acid residues aspartic acid-serine (residues 51-52), glutamine (residue 111), lysine (residue 239), and deletion of glutamic acid (residue 77), and tryptic

peptide T-10 (Baker et al, 1977, residues 77-81) in the sequence. The reason for the differences in the primary structure of human A-I remains to be elucidated.

2) Objective:

Isolation and characterization of apoD. ApoD has been recently identified as a cofactor for lecithin cholesterol acyltransferase (LCAT), the enzyme responsible for esterification of plasma cholesterol.

Methods Employed:

ApoD was isolated from delipidated HDL (dl.063-1.2. g/ml) by cellulose-hydroxyapatite chromatography.

Major Findings:

ApoD was purified by cellulose-hydroxyapatite chromatography to electrophoretic homogenity on SDS and urea disc gel electrophoresis. The molecular weight of apoD by SDS gel electrophoresis was 20,000 ± 2,000. Antibodies prepared in goats to apoD gave a single precipitation arc to isolated apoD and plasma samples. Amino acid analysis of apoD revealed a composition similar but not identical to apoD reported by McConathy and Alaupovic (Biochem. 15: 515, 1976). Studies are currently in progress to further characterize apoD, including the carbohydrate content, and molecular properties of the isolated apolipoprotein.

3) Objective:

To develop improved methods for the automated degradation of peptides and proteins. During the last several years there has become an increasing demand for the development of improved methods for the sequence analysis of polypetides and proteins. Of particular importance has been the difficulty in the sequence analysis of large proteins (>250 residues) due to the inability to add sufficient protein (limit 5 mg) to the Beckman sequencer for adequate yields of PTH amino acids for identification. In addition, the 5 mg protein limit in the Beckman sequencer has limited the length of a sequence which can be determined during a single run. The difficulty in the sequence analysis of large proteins, and the sample size limitation for extended degradations could be alleviated by design changes in the currently available commercial instrument.

Methods Employed:

A prototype sequencer cup with a two fold greater capacity was designed by the MDB Staff, and constructed at Beckman Instruments, California.

Major Findings:

New programs were developed and automated degradations were able to be preformed with the large cup without difficulty in the production model

Beckman sequencer. The protein load was able to be increased from 5 mg to 12-14 mg per sequencer run. Proteins of >250 residues have been degraded without difficulty. In addition, sufficient material was able to be added to the cup to permit degradations on sperm whole myoglobin to be extended from 30-35 to 65-70 residues. Of particular importance was the unexpected finding that degradations with the large cup were associated with much less overlap permitting more definitive identification of the amino acid residues in the sequence. These results represent a major breakthrough in sequence methodology and will be of great use in the sequence of proteins in the future.

Significance:

These studies are directed toward the elucidation of the molecular structure of apolipoproteins and other proteins of importance in biological systems. The determination of the primary structure of the apolipoproteins is a prerequisite for our study of the molecular forces involved in protein-protein and protein-lipid interactions. This information is fundamental to our understanding of the molecular structure of plasma lipoproteins and mechanisms of lipid transport in normal individuals and in patients with disorders of lipid metabolism and atherosclerosis.

Proposed Course:

The isolation, characterization, and structural analysis of the plasma apolipoproteins will be continued with particular emphasis on the apoD, E, and C-II proteins. The isolated proteins will also be investigated with respect to their role as cofactor for enzymes involved in lipid metabolism and vehicles for lipid transport in normal and dyslipoproteinemia subjects.

Publications

1. Gwynne, J. T., Mahaffee, D., Brewer, H. B. Jr., and Ney, R. L.: Adrenal cholesterol uptake from plasma lipoproteins: regulation by corticotropin. Proc. Natl. Acad. Sci. U.S.A. 73: 4329-4333, 1976.

2. Osborne, J. C. Jr. and Brewer, H. B. Jr.: Plasma lipoproteins. Adv. Prot. Chem. 31: 253-337, 1977.

3. Brewer, H. B. Jr. and Bronzert, T.: Human plasma lipoproteins. Fractions 1: 1-11, 1977.

4. Brewer, H. B. Jr., Thomas, F., LaRue, A., Ronan, R., Houser, A., and Bronzert: The primary structure of human A-I. Biochem. Biophys. Rec. Comm., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02011-02 MDB

PERIOD COVERED
July 1, 1976 thrugh September 30, 1977

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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Other:	H. Bryan Brewer, Jr., M.D.	Chief	MDB	NHLBI
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	Anne Houser, B.S.	Chemist	MDB	NHLBI
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COOPERATING UNITS (if any) Joel Moss, M.D., Martha Vaughn, M.D., Laboratory of Cellular Metabolism, NHLBI; Peter H. Fishman, Ph.D., Roscoe O. Brady, M.D., Developmental and Metabolic Neurology Branch, NINCDS; Silvestro Formisano, Ph.D., Clinical Endocrinology Branch, NIAMDD.

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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)

The molecular properties of apolipoproteins have been shown to be quite sensitive to numerous experimental conditions, including protein concentration, solvent composition, pH, temperature and pressure. Apolipoproteins A-I, A-II, and C-I each self-associate in aqueous solution with major changes in secondary, tertiary and quaternary structure. In addition, the molecular properties of apoC-III have been found to depend strongly on carbohydrate content. It is inferred from these data that one of the control points in the molecular interactions and metabolism of plasma lipoproteins is the specific conformation of apolipoproteins in lipoproteins and lipoprotein particles. These and other experimental observations have been used to form a framework for the evaluation of lipid-lipid, lipid-protein and protein-protein interactions in lipoproteins and lipoprotein particles. A knowledge of these forces is fundamental to our understanding of lipid transport and metabolism in normal and abnormal states.

Project Description:

1) Objective:

Evaluation of the role of apolipoproteins in the quaternary organization of HDL. The first phase of this study has centered on the major apolipoproteins in HDL, apoA-I and apoA-II. It has been shown that these apolipoproteins self-associate in aqueous solution and that their secondary, tertiary and quaternary structure depends quite strongly on protein concentration and solvent composition. Since the binding of ligands, such as phosphatidyl choline, also causes changes in the secondary and tertiary structure of apoA-I and apoA-II, a knowledge of the molecular species in solution, and their corresponding structure, is a prerequisite for the quantitative evaluation of ligand affinity and/or binding capacity. The molecular properties of apoA-II as a function of protein concentration, solvent composition and temperature are well defined. In contrast, although several laboratories have extensively investigated apoA-I, its molecular properties in aqueous solution are still only poorly defined. Since a knowledge of the molecular properties of apoA-I is critical to the quantitative evaluation of the quaternary structure of HDL, we have reevaluated the molecular interactions of apoA-I in aqueous solution.

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 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 on 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:

In agreement with previous studies, apoA-I, isolated from HDL was found to undergo reversable self-association in aqueous solution with concomitant changes in secondary structure. With decreasing protein concentration, and hence dissociation, a 20% decrease in secondary (i.e., α-helical) structure was observed. The association of apoA-I was found to be sensitive to ionic strength and pressure; the apparent weight average molecular weight increased with increasing ionic strength and decreasing pressure (decreasing rotor speed in the ultracentrifuge). The data were consistent with a monomer-dimer-tetramer-octamer association scheme if a change in partial specific volume of 5.5×10^{-2} ml/gm upon association is postulated. The effects of ionic strength and pressure account for the divergent data regarding the molecular properties of apoA-I reported in the literature. More importantly, these studies indicate that the methods normally used to isolate lipoprotein

particles, i.e., in the presence of high salt and high pressures, require a thorough examination. If the intermolecular forces in lipoprotein particles are the same as those involved in apolipoprotein self-association, presumably hydrophobic in both cases, then the quaternary structure of lipoprotein particles may be perturbed during fractionation and purification.

2) Objective:

Evaluation of the mechanism of VLDL-HDL exchange of the C-apolipoproteins and the role of these apolipoproteins in the quaternary structure of VLDL and HDL. The first phase of this investigation is an evaluation of the properties of the apolipoproteins in aqueous solution. As summarized in the previous report, apoC-I has been shown to undergo reversable self-association in aqueous solution with dramatic changes in secondary structure. With dissociation of the oligomers of apoC-I there is a concommitant 50% decrease in secondary structure. ApoC-I is monomeric below pH 3 and the apparent molecular weight versus concentration profile at neutral pH is consistent with a monomer-dimer-trimer association scheme. These dramatic changes in the secondary structure of apoC-I upon association would appear to indicate that intramolecular, as well as intermolecular, interactions may be involved in the VLDL-HDL exchange of the C-apolipoproteins. The molecular properties of apoC-III are currently under investigation.

Methods Employed:

Those methods outlined under objective (1) above.

Major Findings:

Preliminary studies of apoC-III indicated that the molecular properties of this apolipoprotein were drastically dependent upon carbohydrate content. ApoC-III can be isolated in 3 forms, C-III_0, C-III_1, and C-III_2 containing 0, 1 and 2 moles of sialic acid respectively per mole of apolipoprotein. The molecular weight versus concentration profile of each of these forms, obtained by conventional sedimentation equilibrium experiments, is consistent with a reversable self-association of these apolipoproteins. However, in each case the lower limiting molecular weight was much lower than the known monomer molecular weight of apoC-III. In view of the known anomalous behavior of glycoproteins in the model E ultracentrifuge and the absence of detectable proteolytic activity or low molecular weight contaminants, this investigation was expanded to include a sample of apoC-III which had been β-eliminated to remove the carbohydrate moiety. The β-eliminated material was well behaved in the ultracentrifuge and the limiting molecular weight was consistent with that expected from the amino acid sequence of apoC-III. All evidence is consistent with the anomalous behavior in the ultracentrifuge being due to the carbohydrate moiety. More recently investigations have been directed at evaluating the molecular weight of apoC-III as a function of protein concentration by laser light scattering. Comparison of the techniques, i.e., ultracentrifugation and light scattering, should yield valuable insight into not only the molecular properties of apoC-III, but also into the ultracentrifugal behavior of glycoproteins in general.

3) Objective:

Comprehensive analysis of the implications of recent experimental observations regarding the molecular interactions and metabolism of plasma lipoproteins. Research on plasma lipoproteins has expanded rapidly over the last decade, resulting in a dramatic increase in our knowledge of the quaternary structure, intermolecular interactions and metabolism of plasma lipoproteins. These recent advances have necessitated a critical analysis of the interpretation of many previous studies. In addition, the increasingly apparent heterogenity of lipoprotein particles has resulted in a nomenclature that is imprecise and often ambiguous. The recent literature has been critically reviewed and a general concept of, and a nomenclature for, plasma lipoproteins has been developed which encompasses the current knowledge in the field.

Major Findings:

The available data in the literature have been analyzed in terms of a concept in which lipid-apolipoprotein, lipoprotein-lipoprotein and lipoprotein-membrane interactions in plasma are freely reversable and governed entirely by the laws of mass action. Viewed in this manner, the existance of a lipoprotein particle is governed by the concentration of the individual components and their affinity for one another. Under the framework of this model the isolation of an apolipoprotein, or any other component of plasma lipoproteins, in a given fraction, and hence any given density range, does not necessarily mean that this apolipoprotein does not form complexes with other plasma lipoproteins, with different densities, in plasma. Conversely, the absence of a given apolipoprotein in an isolated fraction does not necessarily mean that this apolipoprotein does not form complexes with plasma lipoproteins, present in the isolated fraction, in plasma. A nomenclature has been developed which allows a more precise identification of plasma lipoproteins present in plasma and in isolated fractions of plasma.

4) Objective:

Conversion of the chromatrix laser light scattering photomer to aqueous systems. The determination of molecular weights and changes in molecular weight as a function of solvent composition and ligand interaction is fundamental to the quantitative evaluation of the quaternary structure and molecular interactions of apolipoproteins, lipoproteins and lipoprotein particles. Molecular weights are routinely obtained with the Spinco Model E ultracentrifuge. Although in many cases the model E is the method of choice for determination of molecular weights, the technique is time consuming and it is therefore inconvenient to monitor the effects of perturbants, such as the presence of ligands or changes in solvent composition by ultracentrifugation. In addition, if the interactions being investigated are pressure dependent, the results of sedimentation equilibrium experiments are difficult to interpret. Light scattering offers a viable alternative and the use of a laser light source allows molecular weight measurements in the concentration range needed for evaluation of the molecular interactions of plasma lipoproteins. Commercial instruments are designed primarily for non-aqueous systems, however the conversion to aqueous systems is, in principle, straight forward.

Major Findings:

With the present instrumentation, molecular weights can be obtained at concentrations approximately an order of magnitude lower than that required for conventional light scattering photometers (approximately 0.15 mg/ml for a protein of molecular weight 25,000). The method requires approximately 1.5 ml of solution and can be performed in approximately 45 minutes. Evaluation of a modified system capable of measurements with smaller volumes, 0.3 to 0.5 mls should be complete within the next few months. Measurements of the molecular weight of apoA-I at atmospheric pressure were, within experimental error, equal to those predicted from sedimentation equilibrium experiments. The use of the laser light scattering photometer in conjunction with the model E will give the versatility required for efficient evaluation of molecular weight.

5) Objective:

Evaluation of the molecular mechanism by which choleragen interacts with cell membranes. Choleragen is composed of two subunits: the A subunit is believed to activate adenylate cyclase whereas the B subunit, or binding subunit, appears to interact specifically with a membrane surface receptor, which is believed to be the monsialoganglioside G_{M1}. An evaluation of the structural determinants for, and the perturbations induced by, the interaction between choleragen and G_{M1} should yield valuable insight into ligand receptor interactions in general. Studies with the intact ganglioside are complicated by the self-association of G_{M1}. However, the oligosaccharide moiety of G_{M1} is believed to be responsible for the specific interaction between choleragen and its receptor. Since this moiety does not form micelles in aqueous solution, initial probes into the mechanism of cholergen-receptor interactions were performed with the oligosaccharide moiety of G_{M1}.

Methods Employed:

Those methods outlined under objective 1) above.

Major Findings:

The oligosaccharide moiety of G_{M1} was shown to interact specifically with the B subunit, but not the A subunit of choleragen, inducing changes in tryptophanyl fluorescence similar to those previously reported for G_{M1}. Equilibrium dialysis, gel chromatography, and spectral measurements were consistent with an affinity of 1.4×10^7 l/mole. Circular dichroic spectra were consistent with a high content of β pleated sheet structure in choleragen and the isolated A and B subunits. In addition to perturbations in tryptophanyl fluorescence, the binding of oligosaccharide also induces a structural rearrangement of choleragen and the B subunit, as evidenced by changes in circular dichroism. The multivalent nature of choleragens interaction with G_{M1}, and the resulting conformational changes may be indirectly or directly involved in the activation of adenylate cyclase.

Significance:

This work is directed toward a more complete understanding of the physicial properties and functions of the plasma apolipoproteins. A detailed knowledge of the molecular properties of the apolipoproteins is a prerequisite to our understanding of protein-protein and protein-lipid interactions. The elucidation of the molecular forces, and quaternary structure of plasma lipoproteins will be of fundamental importance in our ultimate understanding of the mechanism of lipid transport in normal individuals and in patients with disorders of lipid metabolism and atherosclerosis.

Proposed Course:

During the next year the detailed analysis of the molecular properties of the plasma apolipoproteins will be continued. Particular attention will be directed toward the analysis of C-III, E, and D apolipoproteins. The molecular properties of A-I and A-II have now been completed, and studies on A-I and A-II interaction will be initiated. An understanding of the molecular properties of A-I and A-II will also now permit more definitive studies on lipid-protein interaction. These studies will significantly increase our understanding of the molecular forces involved in lipid-protein interaction.

Publications:

1. Osborne, J. C. and Brewer, H. B., Jr.: The plasma lipoproteins. Advan. Protein Chem. 31: 253-337, 1977.

2. Moss, J., Osborne, J. C., Fishman, P., Brewer, H. B., Jr., Vaughan, M., and Brady, R. O.: Effect of gangliosides and substrate analogues on the hydrolysis of nicotinamide adenine dinucleotide by choleragen. Proc. Natl. Acad. Sci. U.S.A., 74: 74-78, 1977.

3. Osborne, J. C., Bronzert, T., and Brewer, H. B., Jr.: Self-association of apoC-I from the human high density lipoprotein complex. J. Biol. Chem., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02012-02

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Purification and Characterization of HMG-CoA Reductase and Its Role in the Regulation of Cholesterol Metabolism

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

PI:	Zafarul Beg, Ph.D.	Visiting Scientist	MDB NHLBI
Other:	H. Bryan Brewer, Jr., M.D.	Chief	MDB NHLBI
	John Stonik, B.S.	Chemist	MDB NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Molecular Disease Branch

SECTION
Peptide Chemistry Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.25	1.25	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Chicken 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA reductase), the rate limiting enzyme in cholesterol biosynthesis, has been purified to homogenity from liver microsomes by agarose-blue dextran chromatography. The enzyme was a single band on electrophoresis, and had a monomer molecular weight by SDS electrophoresis of 18,000. A monospecific antibody to chicken HMG-CoA reductase was developed in goats. The chicken antibody demonstrated cross reactivity with rat liver, but not human fibroblast HMG-CoA reductase.

Chicken liver HMG-CoA reductase solubilized from liver microsomes could be rapidly inactivated by ATP-Mg^{++} suggesting that phosphorylation of the enzyme was associated with loss of enzymatic activity. Treatment of the modified enzyme with a cytosolic fraction rich in phosphatase activity rapidly restored enzyme activity. These results are consistent with the concept that phosphorylation-dephosphorylaton may be an important cellular mechanism for control of enzyme activity.

Project Description

1) Objective:

Isolation and characterization of chicken liver 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate limiting enzyme of hepatic cholesterogenesis.

Methods Employed:

Microsomal pellets were isolated from homogenized chicken livers by continuous-flow centrifugation. This new centrifugation technique provided a method for large scale preparation of liver microsomes for enzyme isolation. A new freeze-thaw technique for the solubilization of HMG-CoA from liver microsomes was developed. Frozen microsomes (-20°C) were thawed at room temperature and homoginized in a KH_2PO_4-sucrose buffer (pH 7.2) and centrifuged (100,000 xg, 60 min.). The solubilized enzyme was purified to homogenity by heat treatment (65°C, 10 min.), followed by affinity chromatography on agarose-blue dextran.

Major Findings:

HMG-CoA reductase has been purified to homogenity from chicken liver microsomes. The isolated enzyme was a single electrophoretic band on polyacrylamide gel electrophoresis (PAGE), SDS-PAGE, and PAGE in 6M urea. The monomer molecular weight by SDS-PAGE was 18,000, which is significantly smaller than the 50-60,000 molecular weight reported for rat and yeast HMG-CoA reductase. The enzyme had a pI of 6.7 ± 0.2. The enzymatic activity associated with HMG-CoA reductase was eluted from the single electrophoretic band observed on thin layer isoelectric focusing and PAGE verifying that the enzymatic activity was assoicated with the electrophoretic band ascribed to HMG-CoA reductase. The development of a procedure for the large scale isolation of HMG-CoA reductase will now permit a detailed characterization of the physical chemical, structural, and molecular properties of this important cellular enzyme.

2) Objective:

Development of an antibody to chicken liver HMG-CoA reductase.

Methods Employed:

Purified chicken HMG-CoA reductase was injected into multiple irtradermal sites in a goat.

Major Finding:

An antibody with high affinity for chicken HMG-CoA reductase was obtained. The antibody showed a single precipitant arc with microsomal, solubilized, and purified enzyme. The antibody raised against chicken reductase cross-reacted with rat hepatic, but not human fibroblast reductase. A quantitative rocket electroimmunoassay is currently being developed which will permit the

quantitation of HMG-CoA reductase in tissues under a number of different physiological conditions.

3) Objective:

Analysis of the modulation of chicken liver HMG-CoA reductase by ATP-Mg^{++}.

Methods Employed:

Microsomal, solubilized, and purified HMG-reductase was incubated with ATP-Mg^{++} and cytosolic fractions obtained by ammonium sulfate fractionation.

Major Findings:

Microsomal and solubilized HMG-CoA reductase was rapidly inactivated following incubation with ATP-Mg^{++}. Purified enzyme could be inactivated with ATP-Mg^{++} if a cytosolic fraction rich in protein kinase (0-30% ammonium sulfate fraction) was added to the incubation media. HMG-CoA reductase activity could be restored following incubation with a fraction rich in phosphatase.

The results are consistent with the concept that phosphorylation-dephosphorylation may be an important cellular method for the regulation of enzyme activity. Studies utilizing ATP-γ-P^{32} are currently underway to definitively demonstrate phosphorylation-dephosphorylation of the purified enzyme.

4) Objective:

Evaluation of factors influencing the catalytic rate of HMG-CoA reductase.

Methods Employed:

The enzymatic activity of HMG-CoA reductase was assayed as a function of pH, temperature, cofactor requirements, and ligands.

Major Findings:

The pH optimum for enzyme activity was shown to be pH 6.75. Enzymatic activity was stable to 65°C. A decrease in activity observed with temperature greater than 65°C was attributed to thermal denaturation of the enzyme. Thermal denaturation of reductase at 65°C could be significantly decreased by the addition of bovine serum albumin, KCl or NADPH, to the incubation media. Optimal conditions for the assay of the enzyme were determined and included a pH of 6.75, bovine serum albumin (18 mg/ml), dithiothreitol (2MM), and KCl (0.675M). Coenzyme A, acyl CoA esters, and 3-hydroxy-3-methylglutaric acid and inhibited HMG-CoA reductase activity. The K_M for the D isomer of HMG-CoA was 1.5 µM.

Significance:

These studies are directed toward an improved understanding of the control of cellular cholesterol metabolism. An elucidation of the structure and mode of regulation of the rate limiting enzyme, HMG-CoA reductase, will permit a more detailed analysis of the factors regulating cholesterol biosynthesis.

These results will ultimately provide additional insight into the control of lipid biosynthesis and transport in normal and dyslipoproteinemic subjects.

Proposed Course:

A systematic investigation of the structure, physical chemical properties, and modes of regulation of HMG-CoA reductase will be continued. The modulation of enzyme activity will be studied in normal rats, and in rats fed cholestyramine or cholesterol, injected with insulin/glucagon, or starved. Short term regulation of enzyme activity by allosteric control, and covalent modification will continue to be investigated during the next year. The purification scheme developed for isolation of reductase from chicken liver will be extended to other species including the rat. Isolation and characterization of HMG-CoA reductase from several species will increase our understanding of the role of this enzyme in the regulation of cholesterol biosynthesis.

Publications

1. Beg, Z. H., Stonik, J. A., and Brewer, H. B., Jr.: Solubilization of HMG-CoA reductase from rat and chicken liver microsomes. Anal. Biochem., in press.

2. Beg, Z. H., Stonik, J. A., and Brewer, H. B., Jr.: Purification and characterization of HMG-CoA reductase from chicken liver. FEBS Letters, in press.

2. Beg, Z. H., Stonik, J. A., and Brewer, H. B., Jr.: Catalytic and immunochemical properties of HMG-CoA reductase from Avian liver. J. Biol. Chem., to be submitted.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02014-01 MDB

PERIOD COVERED
July 1, 1976 thrugh September 30, 1977

TITLE OF PROJECT (80 characters or less)
Human Plasma Lipoproteins and Apolipoproteins: Isolation, Quantitation and Metabolism

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

PI:	H. Bryan Brewer, J. M.D.	Chief	MDB	NHLBI
Other:	Thomas J. Bronzert, B.S.	Chemist	MDB	NHLBI
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COOPERATING UNITS (if any) Pierre Alaupovic, Ph.D. and Walter McConathy, Ph.D.
Oklahoma Medical Research Foundation, Oklahoma City, OK.

LAB/BRANCH
Molecular Disease Branch

Cc
Peptide Chemistry Section

INSTITUTE AND LOCATION
NHLBI-NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.25	.25	3.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A new micromethod for the rapid, reliable fractionation of plasma lipoproteins, and quantitation of their cholesterol content has been developed. This method requires 350 µliter of plasma, and can be completed in 3 hours. This method should facilitate the measurement of cholesterol in lipoprotein fractions by routine clinical laboratories.

Techniques for the quantitation of apolipoproteins are under development. Measurements of apoA, C, D, E, and B have been completed in 6 patients with Tangier disease, and indicate a heterogenity of apolipoprotein levels. These results are consistent with the possibility that patients with Tangier disease may have different metabolic defects.

Project Description:

Objective:

Develop a micromethod for the rapid, reliable fractionation of plasma lipoproteins, and quantitation of their cholesterol content.

Methods Employed:

A prototype enzymatic-oxygen electrode cholesterol analyzer, table top air driven ultracentrifuge, and centrifuge tube aspirator were developed by Beckman Instruments, California, in collaboration with the Molecular Disease Branch Staff.

Major Findings:

A new micromethod for the fractionation of plasma lipoproteins and quantitation of their cholesterol content was developed. This procedure requires 350 µliters of plasma, and can be completed within 3 hours. Cholesterol was quantitated by use of a new enzymatic-oxygen electrode analyzer. Correlation coefficients between cholesterol values for plasma from normal and hyperlipidemic individuals obtained with the new analyzer and the Technicon Autoanalyzer II and Smac systems were 0.977 and 0.973, respectively. Plasma lipoproteins were fractionated by centrifugation at plasma and density 1.060 g/ml in a new air driven ultracentrifuge in 2 hours. Lipoprotein density fractions were separated by a prototype air aspirator, and the the cholesterol content determined by the cholesterol analyzer. The cholesterol content of fractionated plasma lipoproteins (VLDL, LDL, and HDL) determined by the new micromethod was in excellent agreement with the values obtained by the procedures standardized by the Lipid Research Clinics.

Objective:

Develop methods for the quantitation of human plasma apolipoproteins.

Methods Employed:

Apolipoproteins A-I, A-II, C-I, C-II, C-III, E and D have been isolated in homogenous form by gel permeation and ion exchange chromatography as outlined in the present and previous annual reports. Antibodies to the individual apolipoproteins have been developed in rabbits or goats. Quantitation of each of the apolipoproteins has been performed by electroimmunoassay in our laboratory or in collaboration with Dr. Pierre Alaupovic and staff of the Oklahoma Medical Research Foundation, Oklahoma City, OK.

Major Findings:

The quantitation of all 8 major apolipoproteins is a prerequisite to an improved understanding of the synthesis and catabolism of individual plasma lipoproteins in normal individuals, and patients with disorders of lipid metabolism and atherosclerosis. Compositional analysis of individual plasma

lipoproteins during dietary studies, and drug therapy will expand our knowledge of the factors regulating lipoprotein metabolism. In addition, the ability to study lipoprotein metabolism with radiolabeled apolipoproteins (see accompanying annual report), and determine the plasma specific activity of apolipoproteins labeled with different isotopes will now enable us to study the metabolism of several different apolipoproteins simultaneously in a single study.

The concentration of all 8 apolipoproteins has recently been determined in 6 patients with Tangier disease. Two patients had decreased levels of apoA, apoB, and D, 3 patients had low levels of apoA, C, and E, and 1 patient had low levels of A, but increased levels of apo B, C, and E. Differences in apolipoprotein concentrations suggest that patients with apoA-I, A-II deficiency may have different metabolic disorders.

Objective:

Isolation, characterization, and metabolism of plasma lipoproteins from VLDL, LDL, and HDL density fractions.

Methods Employed:

Plasma lipoproteins (ex. Lp A-I, A-II, Lp B, Lp E, and Lp D) have been isolated from plasma density fractions by hydroxyapatite, lecthin, and/or affinity chromatography. These studies have been performed in collaboration with Dr. Pierre Alaupovic, and staff, Oklahoma Medical Research Foundation, Oklahoma City, OK.

Major Findings:

The classic density fractions of plasma lipoproteins can now be viewed as a polydisperse collection of lipoprotein particles. An ultimate understanding of the metabolism and function of plasma lipoproteins requires the isolation, characterization and turnover of these individual plasma lipoproteins. Plasma lipoproteins isolated from these density fractions are currently being studied with regard to their ability to regulate cholesterol biosynthesis in fibroblasts from normal and dyslipoproteinemic subjects. Recent studies with LpB, and LDL (LpB, C, E) lipoproteins in normal fibroblasts have shown that both lipoprotein particles are able to bind to fibroblasts, and regulate HMG-CoA reductase. These observations indicate that the lipoprotein particles containing the B protein as the single apolipoprotein can bind to the high affinity receptor process, and regulate HMG-CoA reductase. The E apolipoprotein is, therefore, not a necessary component for LDL cellular interaction.

Significance:

This work is directed toward a more complete understanding of the plasma lipoproteins and apolipoproteins and their role in lipid metabolism and atherosclerosis. The fractionation of lipoprotein particles from classic density fractions will facilitate a more detailed study of the factors involved in lipoprotein synthesis and degradation.

Proposed Course:

Detailed characterization of lipoprotein particles from normal individuals and patients with disorders of lipoprotein metabolism will be continued. Of particular importance are the studies of LpA-I, A-II, LpE, and LpD with respect to their role in the cellular regulation of cholesterol synthesis and efflux, and lipoprotein synthesis and degradation.

Publications:

1. Bronzert, T. J. and Brewer, H. B. Jr.: The quantitation of cholesterol in fractionation plasma lipoproteins: a new micromethodology. Clin. Chemistry, in press.

2. Brewer, H. B. Jr. and Bronzert, T. J.: Human plasma lipoproteins. Fractions 1: 1-11, 1977.

3. Osborne, J. C. Jr. and Brewer, H. B. Jr.: Plasma lipoproteins. Adv. Prot. Chem. 31: 253-337, 1977.

Annual Report of the
Laboratory of Molecular Hematology
National Heart, Lung, and Blood Institute
July 1, 1976 - September 30, 1977

The Molecular Hematology Branch, which has had as its goal the treatment of human genetic diseases (specifically, thalassemia and sickle cell anemia) has, during the past year, been divided into two separate groups: the Laboratory of Molecular Hematology and the Clinical Hematology Branch. The two groups are closely interrelated and continue to collaborate on a number of joint projects. In general, the Laboratory of Molecular Hematology is devoted towards study of basic molecular mechanisms relating to gene expression and protein synthesis, while the Clinical Hematology Branch is oriented more towards programs leading to direct clinical benefit to patients. The Laboratory of Molecular Hematology is composed of two segments: the Unit of Molecular Genetics, which is primarily concerned with the molecular control of eukaryotic gene expression, and the Unit of Protein Biosynthesis, which is primarily concerned with the mechanism and regulation of hemoglobin synthesis at the translational level.

UNIT OF MOLECULAR GENETICS

The immediate objectives of this Unit are to: (1) identify, isolate and characterize the regulatory factors controlling the expression of the globin genes, and (2) to identify, isolate and sequence the regulatory regions of animal and human DNA which are involved in the control of the expression of the globin genes. The information from these studies would be used to study the expression of the globin genes in normal human and in thalassemic DNA. The long term goal is to develop means whereby globin gene expression can be corrected in patients with β thalassemia.

Regulatory factors controlling the expression of the globin genes are being sought by a combination of cell biology and molecular biology techniques. Somatic cell hybrids are formed by virus-induced fusion of human or animal cells with mouse erythroleukemia (MEL) cells. The MEL cell is an established cell line capable of being induced to make hemoglobin. By analyzing for the expression or extinction of hemoglobin synthesis in the various hybrid cells, evidence for positive or negative regulatory factors can be obtained. The laboratory has succeeded, during the past year, in demonstrating that a negative diffusable regulatory factor exists in the cytoplasm of non-erythroid cells. This factor(s), when inserted into MEL cells by the technique of cybridization, produces an inheritable change in the genome of the MEL cell (stable for over 100 generations) such that it can no longer be induced to synthesize hemoglobin. In addition, evidence has been obtained that a positive regulatory factor might exist in a stable, newly isolated variant MEL cell containing a double complement of chromosomes (namely, a 2S MEL cell). It is now being determined if this putative positive factor(s) is capable of inducing hemoglobin in non-erythroid cells. Attempts will be made to isolate, purify and characterize each of these factors. The assay procedure to be used in isolating and purifying the factors involves microinjection of test material into target cells followed by single cell analysis for globin mRNA or hemoglobin synthesis.

In order to determine the mechanism whereby the putative regulatory factors influence globin gene expression, the regulatory sequences contained in chromosomal DNA which control the globin genes are being sought. Full-size globin genes have been successfully synthesized by use of the enzymes RNA-directed DNA polymerase and T4 DNA polymerase. Reaction conditions were optimized for fidelity of transcription as well as for full-size material. This enzymatically synthesized globin gene will be cloned by use of bacterial plasmids and then will be used in an attempt to identify fragments of chromosomal DNA which contain not only the globin structural genes but, in addition, regulatory sequences controlling the expression of the globin structural genes. Interactions between regulatory factors and regulatory sequences can then be studied in cell-free systems, and, in addition, in intact cells by microinjection of DNA and/or factors into target cells.

UNIT OF PROTEIN BIOSYNTHESIS

The goals of this Unit are: (1) to identify and purify the protein and nucleic acid components required for the biosynthesis of α and β globin chains; (2) to establish the step-by-step mechanism whereby the components of (1) are utilized; (3) to identify and purify protein or nucleic acids which appear to regulate protein biosynthesis; (4) to identify the mechanism whereby these regulatory components exert their effect on protein biosynthesis.

At this point, much of goal (1) has been reached; the preparation of homogeneous eIF-1 and eIF-4B appear to represent the remaining task since the other initiation and elongation factors have been purified. The second goal, an understanding of the sequential utilization of protein and nucleic acid components, is currently under study using radiolabeled proteins and nucleic acids. The results from these studies indicate that the initiation factors identified by this laboratory are not ribosomal proteins, but rather proteins which only have a transitory interaction with 40S ribosomal subunit and are cleared from the 40S subunit prior to the joining of the 40S and 60S subunits. For several of the initiation factors (eIF-4C,4D,5) it has not been possible as yet to observe stable binding to either the 40S or 60S subunit although indirect evidence would favor an interaction with the 40S subunit. In addition, studies with several non-NIH collaborators have indicated that eIF-4A and eIF-4B are the major initiation factors involved in the preferential selection of mRNAs for translation.

With respect to the regulation of protein synthesis, there have been some observations which implicate protein kinases and protein phosphatases. In a collaborative study, these proteins, present in reticulocyte lysates, are being purified and their substrate specificity being examined. Additional areas of protein synthesis which may exhibit regulation are being or will be explored (such as the regulation of protein synthesis during development and during the response of an interferon treated cell to viral infection). Finally, based upon the electrophoretic mobility of some proteins in either one or two dimensional gel systems, there is some evidence to suggest that either initiation or elongation factors may play an additional regulatory role in cells, as has been suggested for EF-Tu and $fMet\text{-}tRNA_f$ in E. coli.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02201-05 MH

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Mechanism of Hemoglobin Biosynthesis in Cell-Free Systems

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

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	W. Kemper	Chemist	MH	NHLBI
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	J. Osborne	Staff Fellow	MDB	NHLBI

COOPERATING UNITS (if any) H. Weissbach, Roche Institute of Molecular Biology; B. Thach, Dept. of Biochemistry, Washington University; D. Shafritz, Albert Einstein College of Medicine; J. Traugh, Dept. of Biochemistry, University of California (Riverside); J. Goldstein, The New York Blood Center.

LAB/BRANCH
Laboratory of Molecular Hematology

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
8.0	4.5	3.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The initiation factors required for the translation of globin mRNA have been prepared in pure form except for eIF-4B which has been prepared in a form that appears to be approximately 70% pure. Attempts to further fractionate the one crude component (a mixture of aminoacyl-tRNA synthetases) used in the translation of globin mRNA suggest that additional components may stimulate the synthesis of α and β globin chains. Radiolabeled initiation factors have been prepared by reductive alkylation using [^{14}C] HCHO and $NaBH_4$. The use of radiolabeled initiation factors, AUG, [^{3}H]Met-tRNA$_f$, 40S subunits, 60S subunits, GTP and various GTP analogs have allowed us to define more precisely the sequence of events in AUG directed 80S initiation complex formation.

Based upon the observations that suggest that the hemin controlled repressor (HCR) is a protein kinase, several protein kinases have been isolated from rabbit reticulocytes. These protein kinases have been shown to differentially phosphorylate eIF-2, eIF-3, eIF-4B, and eIF-5. One of the protein kinase preparations appears to have properties similar to HCR as judged by its ability to shut off the initiation of protein synthesis.

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Objectives: The goal of this study is to define the step-by-step mechanism of protein synthesis initiation and to identify components (or events) which lead to regulation at the translational level. The first phase of this study is to identify and purify the entire complement of protein factors required for the initiation of globin mRNA translation. The second phase is to establish the step-by-step mechanism for the utilization of initiator tRNA and mRNA in the factor dependent formation of a functional initiation complex. The third phase is to identify and purify components (proteins or nucleic acids) which appear to regulate physiologically the translation of globin mRNA. The fourth phase is to identify the mechansim of action of components which appear to regulate the initiation of translation of globin mRNA.

Methods: Purification of the individual protein components has been achieved by the use of the following standard biochemical techniques: ion-exchange column chromatography, salt fractionation, gel filtration, preparative gel electrophoresis, sucrose and glycerol density gradient centrifugation, and affinity column chromatography. RNA components (mRNA, crude or fractionated tRNA) were purified by one or more of the following procedures: phenol extraction, gel filtration, reversed phase column chromatography and oligo(dT) cellulose chromatography. The purity and physical properties of the RNA and protein components were determined by quantitative biologic assay combined with gel electrophoresis, sucrose density gradient centrifugation, analytical ultracentrifugation, and amino acid anlysis. Biologic assays were performed with one or more of the following radiolabeled components: initiation factors, RNAs, amino acids, and/or purine ribonucleotides.

Major Findings: (1) An exchange of initiation factors with Drs. Theophil Staehlin and Bernhard Erni indicates that most, if not all, of the initiation factors required to form an 80S complex with initiator tRNA and globin mRNA have been identified, although it is not possible to rule out components which may modulate the rate of protein synthesis initiation (either as a control of the rate of protein synthesis initiation or mRNA discrimination). The cross referencing of the protein factors of our laboratories was the major corner stone in the establishment of the new, unified nomenclature system for eukaryotic initiation factors adopted at the International Symposium on Protein Synthesis held at NIH in October 1976.

(2) The use of radiolabeled initiation factors has allowed the following observations on 40S and 80S initiation complex formation:

A. eIF-3 is capable of binding to 40S subunits in the absence of other components.

B. eIF-2 can bind to 40S subunits in the absence of other initiation factors; however, this binding is dependent upon GTP, Met-tRNA$_f$ and is stimulated several fold by AUG codon.

C. Non-hydrolyzable analogs of GTP (either GDPNP or GDPCP) can satisfy the GTP requirement for Met-tRNA$_f$ and eIF-2 binding to 40S subunits.

D. eIF-3 and eIF-2 reciprocally stabilize the binding of each other to 40S subunits.

E. At limiting levels of eIF-2, the presence of eIF-3 shows a 3-4 fold enhancement of eIF-2 and Met-tRNA$_f$ binding to 40S subunits while at saturating levels of eIF-2, the presence of eIF-3 usually only yields a 50% stimulation in 40S complex formation.

F. In the presence of a hydrolyzable form of GTP, eIF-5 promotes the loss of eIF-2 (and an apparently stoichiometric amount of eIF-3) from 40S complexes. The resulting Met-tRNA$_f$·40S subunit complex appears to be less stable than the parent eIF-2·eIF-3·GTP·Met-tRNA$_f$·40S subunit complex.

G. The formation of an 80S initiation complex requires GTP; non-hydrolyzable GTP analogs do not allow the conversion of 40S complexes to 80S complexes.

H. Studies to date indicate that none of the initiation factors are present in the 80S initiation complex.

(3) Chemical studies on eIF-2 indicate that the most probable structure for this molecule is one which contains a single copy of each of the three polypeptide chains. The resulting molecular weight would be about 125,000 which is in good agreement with the functional molecular weight of 135,000 determined by analysis of 40S subunit complexes containing radiolabeled eIF-2 and [^{3}H]-Met-tRNA$_f$.

(4) Six separate protein kinases have been isolated from reticulocyte 100,000 x g supernatent. These kinases have been shown to phosphorylate eIF-2, eIF-3 and eIF-5 (eIF-4B may also be phosphorylated). The exact biologic function of these kinases is unknown as all the phosphorylated forms of the initiation factors that have been tested were as active as non-phosphorylated forms.

(5) Additional evidence has been obtained that eIF-4A and eIF-4B have an ability to discriminate between various mRNA. eIF-4A and eIF-4B levels influence the ratio of α to β globin chains synthesized in a cell-free protein synthesizing system programmed with rabbit globin ($\alpha+\beta$) mRNA. eIF-4B has the limited ability to reverse the inhibition of protein synthesis initiation caused by analogs of the 5' terminal "cap" structure of eukaryotic mRNAs; this result confirms earlier work using an mRNA binding assay which suggested that one of the mRNA recognition sites of eIF-4B was the 5' terminal "cap."

(6) The α subunit of EF-1 has been isolated in active form. This isolation was made possible by the observation of Kaziro that the activity of the α subunit can only be maintained in the presence of glycerol (at least 10%). The EF-1 α subunit is functional in aminoacyl-tRNA binding to 80S ribosomes and in supporting (with EF-2) poly(U) directed polyphenylalanine synthesis to 10 mM Mg^{++}.

(7) Attempts to raise sheep antibodies against rabbit reticulocyte eIF-2A, eIF-3 and EF-1 have been successful, whereas attempts with eIF-2 have been unsuccessful.

Significance to Biomedical Research and Institute Program:

An understanding of the regulation of gene expression is one of the keys to understanding medical disorders which are due to inappropriate levels (either too high or too low) of enzymes or proteins. While transcriptional control appears to play a major role in effecting these levels, it has become apparent that control at the translational level is also of importance. With a basic understanding of the mechanism of protein biosynthesis, the methods by which translational control are manifested may then be more carefully investigated. In particular, the ability of initiation factors to influence the ratio of α to β globin chain synthesis and the heme deprivation shut-off of globin chain synthesis suggest that some assistance to individuals suffering from some anemias or disorders involving improper balance of α and β globin chain synthesis may be possible. In addition, the ability of interferon to sensitize cells to a dsRNA sensitive shut-off of protein synthesis suggests that controlling protein synthesis during viral infection may be feasible (and thereby limit the severity of viral infection).

Proposed Course of Project: Only the first phase of this project is near completion (purification of factors required for protein biosynthesis). The remaining three phases are only at their beginnings. The mechanisms of protein synthesis initiation utilizing AUG has been nearly completed, but the extension to the utilization of globin mRNA has awaited the availability of radiolabeled eIF-4A and eIF-4B. Phases three and four aimed at identifying the components and regulatory mechanisms of translational control are proceeding but have been slowed by the limited availability of knowledge or materials available from phases 1 and 2. However, based upon the proposed regulation of protein synthesis by protein phosphorylation, collaborative attempts are in progress to identify all of the reticulocyte protein kinases and their physiological substrates. Knowledge of such substrates should then allow an examination of changes in biologic function which accompany protein phosphorylation.

Publications:

1. Kemper, W.M., Merrick, W.C., Redfield, B., Liu, C. and Weissbach, H.: Purification and properties of rabbit reticulocyte elongation factor 1. Arch. Biochem. Biophys. 174: 603-612, 1976.

2. Safer, B., Adams, S.L., Kemper, W.M., Berry, K.W., Lloyd, M. and Merrick, W.C.: Purification and characterization of two initiation factors required for maximal activity of a highly fractionated globin mRNA translation system. Proc. Nat. Acad. Sci. USA. 73: 2584-2588, 1976.

3. Kemper, W.M., Berry, K.W., and Merrick, W.C.: Purification and properties of rabbit reticulocyte initiation factor M2Bα and M2Bβ. J. Biol. Chem. 251: 5551-5557, 1976.

4. Shafritz, D.A., Weinstein, J.A., Safer, B., Merrick, W.C., Weber, L.A., Hickey, E.D. and Baglioni, C.: Evidence for a role of $m^7G^{5'}$ phosphate group in recognition of eukaryotic mRNA by initiation factor M3. Nature 261: 291-294, 1976.

5. Traugh, J.A., Tahara, S.M., Sharp, S.B., Safer, B. and Merrick, W.C.: Factors involved in initiation of hemoglobin synthesis can be phosphorylated in vitro. Nature 263: 163-165, 1976.

6. Twardowski, T., Redfield, B., Kemper, W.M., Merrick, W.C. and Weissbach, H.: Studies on the disaggregation of elongation factor 1 by elastase. Biochem. Biophys. Res. Commun. 71: 272-279, 1976.

7. Golini, F., Thach, S.S., Birge, C.H., Safer, B., Merrick, W.C. and Thach, R.E.: Competition between cellular and viral mRNAs in vitro is regulated by a messenger discriminatory initiation factor. Proc. Nat. Acad. Sci. USA. 73; 3040-3044, 1976.

8. Anderson, W.F., Bosch, L., Cohn, W.E., Lodish, H., Merrick, W.C., Weissbach, H., Wittman, H.G. and Wool, I.G.: International symposium on protein synthesis. FEBS Lett. 76: 1-10, 1977.

9. Safer, B., Peterson, D., and Merrick, W.C.: The effect of hemin contolled repressor on initiation factor functions during sequential formation of the 80S initiation complex. In: Proceedings of the International Symposium on Translation of Synthetic and Natural Polynucleotides Poznan, Poland, 1977, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02205-04 MH

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Mechanism of Action of the Enzyme RNA-directed DNA Polymerase

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

PI:	L.J. Krueger	NIH Predoctoral Fellow	MH NHLBI
	W.F. Anderson	Chief	MH NHLBI
OTHER:	P.J. Kretschmer	Visiting Expert	MH NHLBI
	T.M. Caryk	Chemist	MH NHLBI
	A.F. Thornton	Student Scientist	MH NHLBI

COOPERATING UNITS (if any)

Viral Oncology Branch, NCI

LAB/BRANCH

Laboratory of Molecular Hematology

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.50	1.25	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)

It is the purpose of this project to study the mechanism of action of RNA-directed DNA-polymerase in order to use the enzyme to synthesize full-size complementary DNA and, together with other DNA polymerases, double-stranded DNA. Conditions have been found which permit complete and faithful transcription of globin mRNA into single-stranded complementary DNA and then into double stranded globin gene.

Objectives: RNA-directed DNA polymerase (reverse transcriptase) from avian myeloblastosis virus provides a means for synthesizing a DNA gene directly from isolated messenger RNA. Unfortunately, the quality and quantity of the DNA product have been variable, depending on which mRNA is used as template. It is desired, therefore, to learn the mechanism of action of the enzyme in order to establish optimal conditions whereby a complete (and active) DNA gene can be transcribed from any RNA.

Methods: RNA-directed DNA polymerase, purified by standard procedures from avian myeloblastosis virus, is obtained through the National Cancer Institute. Globin mRNA is isolated from human, rabbit and mouse reticulocytes; DNA product analysis is by formamide polyacrylamide gel electrophoresis. Polyribonucleotides (random copolymers and block copolymers) are synthesized by means of various enzymatic and chemical techniques.

Major Findings:

1) RNA-dependent DNA polymerase isolated from avian myeloblastosis virus (AMV) has been used to prepare DNA complementary (cDNA) to rabbit reticulocyte globin mRNA. The synthetic cDNA transcripts were shown to correspond both in size and composition to the globin mRNA template by the following criteria: (1) size of the cDNA product as estimated by analytic formamide gel electrophoresis; (2) presence of the corresponding 5'-terminal sequence of globin mRNA as assayed by molecular nucleic acid hybridization; (3) base composition of the cDNA as determined by differential radionuclide labeling; (4) double strandedness as analyzed by electron microscopy and S1 nuclease digestion. Sixty percent of the template was copied into cDNA, seventy percent of which was full-sized.

2) Conditions appropriate for the in vitro synthesis of full-size globin cDNA were evaluated to minimize the four competing reactions which result in anomalous transcription: (1) the "slippage" reaction, (2) random initiation, (3) premature termination and (4) second stranding of the cDNA.

3) The full-size cDNA was then purified and used as template for the synthesis of the second strand DNA. A comparative study of DNA polymerase purified from E. coli infected with T4 bacteriophage and AMV DNA polymerase showed the T4 DNA polymerase to be 6-fold more efficient in utilizing the cDNA template and more effective in synthesizing full-size gene as determined by electron microscopy and composite gel electrophoresis. T4 DNA polymerase copied 70% of the input cDNA with a majority of these molecules in a size class consistent with the synthesis of full-size globin gene.

Significance to Biomedical Research and Institute Program: The ability to synthesize a DNA gene in vitro is a major step towards the goal of successful therapy of human genetic diseases. The role of the enzyme itself is important in understanding the pathogenesis of certain cancers.

Proposed Course of the Project: The full-size globin gene synthesized enzymatically in vitro will be cloned by means of bacterial plasmids and used in an attempt to identify DNA regulatory regions involved in globin gene expression.

Publications:

1. Weiss, G.B., Wilson, G.N., Steggles, A.W., and Anderson, W.F.: Importance of full-size complementary DNA in nucleic acid hybridization. J. Biol. Chem. 251: 3425-3431, 1976.

2. Weiss, G.B. and Anderson, W.F.: The problem of nuclease activity in nucleic acid hybridization reactions: theoretical considerations. Biophysical Chemistry 6: 337-344, 1977.

3. Krueger, L.J., Benbow, R.M., Krauss, M.R., Caryk, T.M. and Anderson, W.F.: Quantitative synthesis of full-size globin genes: dependence on template. Nature, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02212-01 MH

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Molecular Control of Eucaryotic Gene Expression

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

PI:	W.F. Anderson	Chief	MH	NHLBI
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	L.M. Lee	Biologist	MH	NHLBI

COOPERATING UNITS (if any)
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LAB/BRANCH
Laboratory of Molecular Hematology

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.85	2.85	2.0

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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. Evidence for the existence of positive and negative regulatory factors in mouse erythroleukemia cells and in non-erythroid cytoplasms, respectively, have been found. Attempts are being made to isolate, purify and characterize these factors.

Objectives: The objective of this project is to isolate the factors involved in the expression of eucaryotic 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. The procedure is to identify those cell types which appear (by cell biology and molecular biology techniques) to contain either positive or negative regulatory factors. Then these cells are fractionated into various components which can be individually tested by microinjection into target cells for their ability to alter globin gene expression.

Methods: Mouse erythroleukemia (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. Resulting cell hybrids are analyzed for the ability to synthesize globin messenger RNA (mRNA) or globin. Globin mRNA is detected by hybridization with the appropriate complementary DNA (cDNA); the presence of globin is detected by radioactive leucine incorporation followed by either electrophoresis or column chromatography. Intact cells are separated into karyoplasts (nuclei) and cytoplasts (enucleated cytoplasm) by centrifugation in the presence of cytochalasin B. Microinjection is carried out either by means of loaded RBC ghosts (see below) or by physical microinjection into specific regions of intact mammalian cells by using ultrathin micropipets.

Major Findings: (1) Evidence for the existence of a diffusable negative regulatory factor in the cytoplasm of non-erythroid cells has been found. When MEL cells are fused with erythroid cells, the resulting cell hybrids can be induced to coexpress the globin genes of both parents. On the other hand, when MEL cells are fused with non-erythroid cells, the resulting cell hybrid cannot be induced to synthesize hemoglobin. In order to determine if the nucleus of the non-erythroid cell is critical for this extinction of hemoglobin expression, cytoplasts (namely, the enucleated cytoplasm) of non-erythroid cells were fused with MEL cells, and the resulting cell cybrids were analyzed for the ability to be induced for hemoglobin. Using certain parental cell lines, these cybrid cells are unable to be induced for hemoglobin and this characteristic is inherited (for over 100 generations). These data indicate that the cytoplasm of non-erythroid cells contains a factor (or factors) which can alter the genetic constitution of erythroid cells in such a way that the expression of the hemoglobin genes is extinguished. Attempts are now being made to identify this factor(s).

(2) Tentative evidence for the existence of a positive regulatory factor has been obtained. As stated above, when (1S) MEL cells are fused with non-erythroid cells, the ability to induce hemoglobin is extinguished. In order to determine if a putative positive regulatory factor existing in MEL cells might be increased by doubling the gene dosage, i.e., by doubling the number of chromosomes present, techniques were developed in order to be able to isolate 2S MEL cells. These cells contain double the normal number of chromosomes and were found to be stable in culture for many months. When 2S MEL cells are fused with non-erythroid cells, many of the resulting hybrids can be induced to make hemoglobin. Studies are now underway to determine if the globin genes on the chromosomes of the non-erythroid cell (in addition to

those on the MEL parental cell) are activated. Attempts will be made to isolate this positive regulatory factor(s) from the 2S MEL cell.

(3) Techniques have been developed to allow microinjection of cell components into intact mammalian cells. For large scale assays (10^6 cells microinjected at one time), RBC ghosts have been employed. Human red blood cells were lysed, incubated in the presence of rabbit globin messenger RNA, resealed, and then fused with Chinese hamster ovary (CHO) cells in an attempt to "microinject" the rabbit globin mRNA into the CHO cell. Conditions were found in which this was successful and the injected mRNA was translated into functionally active hemoglobin. The loaded RBC ghost microinjection technique was also successfully used to inject proteins and DNA into intact mammalian cells. In addition, the technique of physical microinjection of individual cells using ultrathin (1 μm id) micropipets is being developed. Antisera specific for either the rabbit α or β globin chains has been obtained by injecting sheep with purified rabbit α or β globin, respectively. Single cell fluorescent antibody assays using FITC conjugated goat anti-sheep gamma globulin permit the detection of as little as $2x10^6$ rabbit hemoglobin molecules per microinjected cell. In situ molecular hybridization using labelled cDNA can be used to detect mRNA and DNA in single cells. It should be possible by these means to transfer cell components into intact mammalian cells in order to assay for regulatory factors affecting globin gene expression.

Significance to Biomedical Research and Institute Program:

The molecular control of eucaryotic gene expression remains one of the major questions in biology today. Once it is better understood how a gene in a eucaryotic cell is controlled, this knowledge can be applied to a wide range of human diseases including genetic diseases, viral diseases, cancer, etc.

Proposed Course of Project: Fractionation of cell components to identify positive and negative regulatory factors involved in globin gene expression will be carried out. Once identified, the factors will be purified and characterized. The biological function of the individual factors will be studied both in regards to their ability to interact with DNA and their ability to control the regulation of globin gene expression.

Publications:

1. Gopalakrishnan, T.V., Thompson, E.B., and Anderson, W.F.: Extinction of hemoglobin inducibility in Friend erythroleukemia cells by fusion with cytoplasm of enucleated mouse neuroblastoma or fibroblast cells. Proc. Natl. Acad. Sci. USA 74: 1642-1646, 1977.

2. Anderson, W.F., Deisseroth, A., Nienhuis, A.W., Gopalakrishnan, T.V., Huang, A., and Krueger, L.: Cellular and molecular studies on globin gene expression. Third Decennial Review Conference: Cell, Tissue, and Organ Culture. Journal of the National Cancer Institute in press.

3. Axelrod, D.E., Gopalakrishnan, T.V., and Anderson, W.F.: Maintenance of hemoglobin inducibility in somatic cell hybrids of tetraploid Friend mouse erythroleukemia cells and mouse fibroblasts. Somatic Cell Genetics, in press.

Annual Report of the
Clinical Hematology Branch
National Heart, Lung, and Blood Institute
July 1, 1976 - September 30, 1977

The research efforts of this Branch are directed toward understanding the underlying causes and potential treatment of the major red cell disorders. Red cell diseases which produce significant morbidity and mortality in man include thalassemia, severe hemoglobinopathies of which sickle cell anemia is the most common, and the various syndromes of bone marrow failure. Thus various projects in this Branch are directed toward analysis of normal red cell differentiation and maturation, regulation of synthesis of various specific hemoglobins (e.g., fetal and adult hemoglobin in man), and the function of hemoglobin in the transfer of oxygen to tissues. The clinical effects of various anemias as they pertain to organ function are under investigation. Many patients with chronic anemia require regular blood transfusions and ultimately develop pathological iron overload. Efforts are directed towards defining the extent of organ dysfunction due to iron and the potential benefits of iron chelation in these patients.

The Clinical Hematology Branch was established in January of 1977. The Molecular Hematology Branch was divided to form the Clinical Hematology Branch and the Laboratory of Molecular Hematology. This report represents the work initiated within the Section of Clinical Hematology (MHB) and continued after establishment of the Clinical Hematology Branch. Many collaborations continue between individuals in the Clinical Hematology Branch and in the Laboratory of Molecular Hematology.

Hemoglobin is a tetrameric molecule composed of four polypeptides; two are alpha globin and two are either beta or gamma globin. The beta and gamma globins are encoded by closely linked genes on one chromosome, whereas the alpha globin gene(s) lie on a separate chromosome. Expression of gamma globin in fetal life results in the production of fetal hemoglobin (Hb F - $\alpha_2\gamma_2$) whereas expression of the related and closely linked beta globin gene in postnatal life results in the production of adult hemoglobin (Hb A - $\alpha_2\beta_2$). Analogous gamma and beta genes are present in sheep erythroid cells. Certain sheep have an additional beta gene, that for β^C globin. Expression of this gene results in production of Hb C ($\alpha_2\beta^C_2$) in neonatal animals and also in the presence of anemia, hypoxemia, or following injection of erythropoietin. Thus the sheep genes provide an excellent model system to understand differential regulation of the individual closely linked genes and ultimately may provide the basis for development of experimental techniques which can be directly applied to the fetal to adult hemoglobin switch in man.

Two classes of erythroid precursor cells are present in erythroid tissues: 1) the committed stem cells which are present in very small numbers and are not morphologically defined but whose presence is revealed by various colony-forming assays in vitro, and 2) the morphologically defined erythroid precursor cells which include the pro-, baso-, and orthochromatophilic erythroblasts. Our analysis of hemoglobin switching in sheep (Individual Project "Cellular Analysis of Hemoglobin Switching in Sheep") has indicated that commitment with respect to expression of the individual beta and gamma genes occurs within erythroid stem

cells. Thus erythroid colonies generated from sheep bone marrow can be induced to express the beta^{C} globin gene if these cells are exposed at least transiently in vivo to a high concentration of erythropoietin. Once commitment to the expression of a particular gene occurs, the stem cells may develop into mature erythroblasts in the form of colonies in the presence of low erythropoietin concentration and yet retain their commitment to express the beta^{C} gene. Several models can be formulated to explain these cellular phenomenon related to hemoglobin switching in sheep. These include selective mitogenesis of stem cells already committed to express particular genes, modulation of the potential to express the individual genes in a common stem cell pool, or selective expression of particular genes in the progeny of stem cells committed to terminal erythroid maturation at various stages in the developmental sequence between pleuripotent stem cell and proerythroblast. Experiments to test these models include analysis of the specific hemoglobins produced in the colonies derived from various stem cell populations.

Current evidence indicates that maturing erythroblasts are a suitable population for analysis of the molecular components which determine the expression of the particular globin genes. Our study of gene expression in these cells requires specific molecular probes to measure the gene and globin mRNA sequence content in various erythroblast populations. To this end we have prepared complementary DNA specific for the gamma, beta^{A}, beta^{B}, beta^{C}, and alpha sheep globin mRNA sequences (Individual Project "Regulation of Sheep Globin Gene Expression"). Our studies have shown that the individual genes are not deleted or alternatively, amplified in cells in which they are not expressed or expressed, respectively. However, only the globin mRNA sequences of expressed genes are represented in nuclei (or cytoplasm) of erythroid cells. Thus our current notion is that regulation is imposed at the level of chromatin structure or alternatively during transcription. It remains possible that all the genes are transcribed into mRNA sequences found only in very short-lived RNA molecules. Only globin mRNA sequences reflected in hemoglobin produced in the particular erythroid cells would then survive and reach the cytoplasm as mature mRNA molecules.

Further analysis of the molecular basis underlying regulation of the individual globin genes will require more highly purified probes. To this end, double stranded synthetic genes will be re-annealed to plasmid DNA, cloned in bacteria, and the recombinant plasmids containing the individual globin gene sequences will be amplified. This will provide sufficient quantities of highly purified genes for sequence analysis and use as hybridization probes.

Current evidence suggests that chromatin structure restricts the total number of genes expressed in differentiated cells. Thus the Individual Project "Structure of the Globin Genes in Chromatin" is directed toward defining the fine structure of the individual globin genes in chromatin. Currently we are using pancreatic DNAse I as a probe for gene structure. With this method of analysis we have uncovered a striking contrast between the structure of globin genes in normal erythroid cells and in the transformed Friend mouse erythroleukemia (MEL) cells. In normal cells the globin genes are apparently only intermittently open (accessible to panc DNAse) despite the accumulation of globin mRNA, whereas in the MEL cell the globin genes are in the panc DNAse sensitive configuration even in the uninduced state when globin gene

transcription and mRNA sequence concentration is very low. These results point out fundamental differences in globin gene regulation in these two cell populations and illustrate the complex patterns of regulation which globin genes may exhibit in different cell populations. Ultimately these studies will be refined so that the structure of the individual globin genes in sheep chromatin will be determined and structural components responsible for the transcribibility of globin genes will be defined. Similar analyses are being attempted with human bone marrow cells.

Currently we are pursuing two basic experimental approaches to the problem of regulation of individual globin genes. These are: 1) definition of the cellular mechanism of hemoglobin switching as represented by the assays for various stem cell populations, and 2) analysis of the molecular regulatory mechanism in maturing erythroblasts which result in accumulation of specific globin mRNAs. Ultimately our desire is to understand the exact mechanism of interaction of erythropoietin (and other substances) with stem cells to produce commitment to expression of the individual genes. The ensuing molecular events during the development of these stem cells and terminal maturation of their progeny into erythrocytes may ultimately be experimentally defined.

Somatic cell hybrids are being utilized to obtain the chromosomal assignments of the human globin genes (Individual Project "Globin Gene Expression in Somatic Cell Hybrids"). We have found that the human alpha globin gene is on chromosome 16 and the human beta globin gene is on chromosome 11. These assignments also establish the linkage relationship of these genes to certain enzymes, the genes for which are on these chromosomes. It is now possible to select for retention of the human alpha globin gene in hybrid cells formed between normal human erythroid cells and erythroleukemia cells and it may be possible to develop selective systems for retention of the human beta (and gamma) gene. This approach would allow careful analysis of the chromosomal composition and other factors which influence the expression of the individual human globin genes in various hybrid cell populations. (These studies have been performed in collaboration with Dr. Albert Deisseroth of the Section on Experimental Hematology, Pediatric Oncology Branch, NCI).

The thalassemias are congenital anemias characterized by deficient synthesis of one of the globin components of the hemoglobin molecule. In beta thalassemia, a small amount of normal beta globin may be produced, or alternatively, beta globin synthesis may be completely absent. Thus these disorders represent an example of a regulatory mutation. Our analysis is focused on identifying the molecular lesions in these disorders with the anticipation that an insight into normal gene regulation may be achieved (Individual Project "Molecular Defect in Beta Thalassemia"). We have learned that reduced stability of RNA molecules containing beta globin mRNA sequences may underlie the quantitative deficiency of beta globin mRNA found in certain patients with beta$^+$ thalassemia. Direct analysis of the metabolism of various RNA species containing beta globin mRNA sequences may provide further insight into the normal mode of accumulation of the globin mRNA in erythroid cells.

Our analysis of hemoglobin switching and the differential regulation of the individual globin genes are directly applicable to thalassemia and other clinically severe hemoglobinopathies. Reversion to the production of fetal

hemoglobin would be therapeutic since it would compensate for absent or reduced adult hemoglobin in thalassemia and could replace the defective hemoglobin in various hemoglobinopathies.

Many patients with severe congenital or acquired anemias require chronic blood transfusion. Iron accumulation is a life-limiting feature of the transfusion dependent patient. Currently the only therapeutic approach to patients with thalassemia or other severe anemias requiring regular transfusion is iron chelation to avert or reduce the rate of iron accumulation (Individual Project "Iron Chelation in Transfusional Hemosiderosis"). Our studies have focused on identification of various clinical parameters which may be of utility in defining the extent of iron overload. We have found the echocardiogram to be very useful, and using this tool, have uncovered an unanticipated toxicity of ascorbic acid in iron overloaded patients. Several patients exhibited a reversible deterioration in cardiac function while on this medication. Administration of desferrioxamine by the subcutaneous route has proved to be feasible and when used regularly, will prevent further iron overload in most patients who require regular transfusion.

The hemoglobin molecule is highly adapted for its primary function in the transport of oxygen to tissue. Hemoglobin oxygen interaction is therefore fundamental and its manipulation might be of therapeutic importance in various anemias, particularly in sickle cell disease. In this latter disorder, hemoglobin molecules in the deoxy configuration are susceptible to intracellular gelation with resulting deformity and rigidity of individual red cells. This leads to hemolytic anemia and vaso-occlusive crises. By increasing the affinity of SS hemoglobin for oxygen it may be possible to reduce the fraction of molecules in the deoxy configuration and therefore lower the propensity for gelation. However, this might also have a deleterious effect by reducing the amount of oxygen released to tissues. Thus understanding of hemoglobin-oxygen interaction is of fundamental importance in this and other disorders.

Progress toward understanding the respiratory function of blood in oxygen transport has depended on the development of refined methods for measuring hemoglobin oxygen interaction in whole blood or in concentrated hemoglobin solutions (Individual Project "Regulation of the Respiratory Function of Blood"). To this end, equipment has been devised which will measure continuously the oxygen disassociation curve while maintaining the pH and CO_2 tension constant under computer drive control. Data collection and analysis is also facilitated by use of the computer. With this equipment, an accurate formulation of the effect of DPG on the various components of the oxygen disassociation curve has been defined. Several mutant hemoglobins have been characterized with regard to their hemoglobin oxygen affinity. Furthermore we have analyzed the oxygenation properties of Hb C, the particular hemoglobin that appears under situations of erythropoietic stress in goats and sheep. A unique sensitivity to CO_2 is characteristic of this hemoglobin despite the absence of the usual CO_2 binding site at the N-terminal position of the beta chain. These observations provide an opportunity to gain new insight about the interaction of this effector with the hemoglobin tetramer.

Although it is commonly thought that the oxygen affinity of SS blood is markedly reduced, a careful analysis has yielded the unanticipated observation

that this parameter may be easily distorted in the laboratory (Individual Project "Alteration of Blood Oxygen Affinity in the Treatment of Sickle Cell Anemia"). Apparently once deoxygenation of SS cells occurs in vitro, irreversible (or poorly reversible) gelation of Hb S occurs within individual red cells. This geled hemoglobin does not participate in subsequent interaction with oxygen and therefore markedly reduces the measured oxygen affinity of these cells. Only when great care is exercised in removing blood from the individual and in its manipulation in the laboratory, can accurate measurement of oxygen affinity be achieved. With these methods we have learned that hemoglobin oxygen interaction in SS disease is nearly normal. Thus the anemia in these individuals is of greater physiological consequence than previously would have been predicted. Exchange transfusion provides an opportunity to replace SS cells in individual patients with cells capable of normal oxygen transport (Individual Project "Effect of Partial Exchange Transfusion on Oxygen Transport in Sickle Cell Anemia"). Thus direct manipulation of hemoglobin oxygen interaction may be achieved in vivo and its effect on the functional status of the patient may be determined.

The rate of sedimentation of SS red cells is directly related to the oxygen tension. When fully oxygenated, the cells are capable of rouleau formation and rapid sedimentation, whereas deoxygenated and sickled cells do not interact with each other and the sedimentation rate is very low. This parameter may be useful for quantitating the sickling propensity of cells from various patients and also may provide a sensitive screening assay for testing anti-sickling compounds.

The several projects within this Branch are related to the biosynthesis of hemoglobin, the regulation of production of specific hemoglobins in various developmental and experimental states, and the function of hemoglobin in red cells. With this comprehensive approach we hope to provide an experimental basis for an optimal therapeutic approach to various red cell disorders.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 02203-05 CHB

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Molecular Defect in Beta Thalassemia

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
Other:	Patricia Turner, Bio Lab Technician	CHB NHLBI
	E. J. Benz, Jr., Research Associate	CHB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.75	0.25	0.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

These studies are directed toward determining the molecular lesion which is responsible for decreased beta globin synthesis in patients with homozygous beta thalassemia. Current evidence indicates that there is a quantitative deficiency of beta globin mRNA in reticulocytes. This might result because of defective transcription of the globin genes in chromatin, inefficient processing and therefore accumulation of the globin mRNA during erythroid maturation, or reduced stability of the final globin mRNA product. Our studies are directed toward determining, in individual patients, which of these mechanisms is responsible for reduced beta globin mRNA. Thus the spectrum of molecular defects in beta thalassemia will be determined and ultimately the alterations in mRNA synthesis or metabolism will be correlated with the nucleotide sequence of molecules containing beta globin mRNA sequences.

Objectives:

The objective of these studies is to use the naturally occuring model of defective gene regulation provided by beta thalassemia. Patients with this disorder produce a reduced quantity of a normal polypeptide. Recognition of certain molecular lesions in this disease may provide clues as the specific mechanism for regulation of expression of individual genes in human cells. Furthermore, appreciation of the molecular basis of this disorder may provide an approach to specific therapy.

Methods:

1. Bone marrow and peripheral blood reticulocytes are obtained from patients with homozygous beta thalassemia. Separate nuclear and cytoplasmic RNA fractions are obtained after purification of nuclei. In other experiments these cells are initially incubated with [^{3}H]uridine to label the RNA. A chase after the removal of the radioactive uridine provides an opportunity to study the stability of molecules containing globin mRNA sequences.

2. The steady state concentrations of globin mRNA are determined by annealing to appropriately purified alpha, beta, and gamma globin complementary DNA. Alpha globin mRNA is purified from normal human reticulocyte mRNA by polyacrylamide gel electrophoresis. Human beta globin mRNA is obtained from reticulocytes of patients with Hb H disease. Gamma globin cDNA is prepared by utilizing fetal globin mRNA ($\alpha\beta\gamma$) to prepare a mixed cDNA. The gamma sequences are recovered after the mixed cDNA is annealed to adult human mRNA ($\alpha\beta$). The duplexes are removed leaving single stranded gamma globin cDNA.

3. To quantitate the labeled globin mRNA sequences obtained after pulse labeling of erythroid cells, RNA is annealed to nonradioactive cDNA. This may be performed in solution and the amount of radioactive RNA incorporated into hybrid is determined by exposure of the duplexes to pancreatic ribonuclease A and T_1 ribonuclease. Alternatively, cDNA is synthesized on oligo dT cellulose and the specific globin mRNA sequences recovered by affinity chromatography.

Major Findings

Careful study of RNA obtained from nuclei, cytoplasm, and reticuocytes of three patients with β^+ thalasemia has yielded the observation that the beta globin mRNA may be relatively unstable in these cells. Thus the concentration of beta globin mRNA (relative to alpha) was higher in nuclear than in cytoplasmic RNA and the cytoplasmic concentration was higher than that found in peripheral blood reticulocytes. Conversely, gamma mRNA was found in lower concentrations in nuclei than in cytoplasm indicating that its accumulation may be accentuated in the bone marrow cells of these patients. We conclude from these studies that deranged metabolism of RNA molecules containing beta globin mRNA sequences may be responsible for the quantitative deficiency of beta globin mRNA in the cytoplasm of certain patients with homozygous beta thalassemia.

During the past year we have had the opportunity of studying a patient with a moderately severe beta thalassemia syndrome who also had severe arterial hypoxemia due to cyanotic congenital heart disease. Surgical correction of Tetralogy of Fallot resulted in relief of the arterial hypoxemia. After relief of the hypoxemia there was a decrease in the alpha/beta biosynthetic ratio, presumably due to a shift in the kinetics of production of abnormal cells. Of more interest was a marked increase in the production of Hb F. We interpret the latter finding to be due to a relative shift towards maturation of cells committed to Hb F production.

Significance to Biomedical Research and the Institute Program:

Homozygous beta thalassemia is a disease which causes serious morbidity and mortality to its victims. Thus an understanding of the genetic basis of this disorder may result in therapy which would be of extraordinary benefit to these individuals. Furthermore, this disease is a prototype of a regulatory human disorder and may provide insight into the mechanism of gene regulation in human cells.

Proposed Course of the Project:

Subsequent efforts during the next ensuing year will be focused on analysis of the mRNA metabolism in cells of individual patients with homozygous beta thalassemia. Ultimately the sequence of these RNA molecules must be determined. Currently the most feasible means of achieving this desired goal is to prepare double stranded molecules, incorporate these into bacterial plasmids, clone these in bacteria, and amplify the recombinant plasmids to obtain sufficient DNA for convenient chemical sequence analysis.

Publications:

1. Nienhuis, A., Turner, P., and Benz, E.J.,Jr.: Relative stability of alpha and beta globin mRNA in homozygous beta^{+} thalassemia. Proc. Natl. Acad. Sci. USA, 1977. In press.

2. Peterson, D., Winslow, R.M., Adamson, J.W., Morrow, A.G., Nienhuis, A.W.: Tetralogy of Fallot with hypoxemia in a patient with beta thalassemia intermedia. Am. J. Med. 1977, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02204-05 CHB

PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)
Cellular Analysis of Hemoglobin Switching in Sheep

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

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	W. Whitehouse	Chief, Television Engineering Section, PSD	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The purpose of these studies is to establish the basic developmental, cellular and molecular events operating in the regulation of the gene sequences coding for fetal γ and adult β^A and β^C globins synthesized by sheep erythroid cells. The ultimate goal is to provide a biological rational for the therapeutic re-utilization of the human γ gene in disease conditions where defective β globin polypeptide synthesis results in severe anemia. In vivo and in vitro, erythropoietin (ESF) causes a switch from β^A to β^C globin synthesis in adult sheep bone marrow cells. The detailed ontogeny of the β^A to β^C switch is sought in terms of 1) which precursor cells in the erythroid developmental series are phenotypically modulatable by ESF, and 2) whether ESF modulation involves regulating differential transcriptional activity within individual cells comprising a homogeneously pleuripotent population or the selective mitogenesis of precursor sub-populations of cells already committed to predetermined levels of β^A and β^C gene activity. Recent experiments have demonstrated that fetal liver erythroid cells producing only γ globin is stimulated by ESF to synthesize β^C globin in vitro, but not in vivo. This observation provides the impetus for future experiments to identify microenvironmental or hormone-like factors controlling globin phenotype.

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Objectives: The ESF-induced switch in hemoglobin phenotype of sheep erythroid cells grown in plasma clot cultures is the biological model used in this project. Understanding the relationship between cellular differentiation, hemoglobin synthesis, and the regulation of globin gene transcription is being studied. Immediate research objectives can be stated as three questions: 1) What is the detailed ontogeny of erythroid precursor cells? 2) Which cells in the erythroid developmental series are phenotypically modulatable by ESF? 3) Does ESF modulation involve regulating differential transcriptional activity or the selective mitogenesis of precursor populations already committed to predetermined levels of β^A and β^C gene activity.

Methods: To obtain differentiating erythroid cells, bone marrow from normal lambs and fetal livers are removed surgically. Peripheral blood from phlebotomized lambs is used as a source of reticulocytes. Enriched populations of early and late differentiating cells are isolated by isopynic centrifugation in Ficoll-Metrizamide density gradients alone, or in combination with unit gravity velocity sedimentation through BSA gradients. Very homogeneous populations of reticulocytes are isolated by isopynic centrifugation in Ficoll or Stractan density gradients. Cells are cultured in supplemented F-12 medium clotted by the addition of bovine plasma. For microscopic examination and quantitation of colony size and number, plasma clot cultures are dried onto slides and stained with benzidene to identify erythroid colonies. Quantitation of colony size and number is performed by a computer-assisted video analysis which measures the projected areas of benzidene-stained colonies. Globin synthesis in culture is measured by incorporation of ^{3}H-leucine and subsequent ion exchange chromatography. Mass ratios of hemoglobins in reticulocyte populations are determined by isoelectric focusing and quantitative densitometry. The hemoglobin content of individual reticulocytes is estimated by lysis of reticulocytes dispersed in agarose gels containing specific anti-Hb C antiserum, where the density and size of the precipitin sphere that forms is related to the amount of hemoglobin present.

Major Findings:

1) The ontogeny of erythroid differentiation in plasma clot cultures has been modeled by deducing the developmental relationships of early stem cells from the size and number of colonies observed at various times of incubation. The erythroid series can be described as progressive developmental advancement from a small, dividing population of morphologically undefined stem cells to morphologically definitive pro-, basophilic, polychromatophilic and orthochromic erythroblasts. Non-dividing orthochromic erythroblasts mature into reticulocytes and finally circulating erythrocytes. In plasma clot cultures, progeny of dividing cells are mechanically constrained in the distance that they can move apart. Early stem cells, i. e., those cells having the greatest potential number of divisions, are expected to form the largest colonies or bursts of large colonies. Adult sheep marrow and fetal liver cultured for 120 hours yield individual colonies of varying size, large fused colonies, and bursts of closely-spaced, individual large colonies. Video area measurements resolved individual colonies into those containing 2-4, 8, 16, etc. cells/colony. The largest individual colonies observed were 64 cells/colony. Large, fused colonies were

multiples of 64 cells/colony (by extrapolated area), where two 64-cell colonies were most often observed. Bursts of individual colonies were most often groups of four 64-cell colonies. An initial interpretation of these results is that eight cell divisions occur during normal erythroid development. A scheme is proposed where stem cells undergo three divisions (assayable as bursts of colonies, large fused colonies and individual 64-cell colonies, respectively) before becoming morphologically identifiable pro-erythroblasts. The remaining 32- to 2-cell colonies presumably arise from the precursor cells in the definative erythroid series. Isopycnic and velocity centrifugation of erythroid cells prior to culture has established, as expected, that early precursor cells are less dense and greater in volume than late cells.

2) A hypothesis was formulated that a continuous change in Hb C content of individual reticulocytes as a function of ESF stimulation argues for the existence of a homogeneous precursor cell population. A discontinuous change of Hb C content around discrete mean values argues for selective mitogenesis of sub-populations. Pure populations of reticulocytes were isolated from phlebotomized lambs. This approach was utilized to provide a temporally uniform cohort of cells where variations in globin synthesis as a function of developmental advancement can be neglected. In collaboration with Dr. S. Boyer at Johns Hopkins University, attempts were made to determine the Hb C content of individual reticulocytes by micro-immunoprecipitation. The macroscopic Hb C percentage of total hemoglobin in the entire population was approximately 52. Since the phlebotomized animal was in a quasi-steady-state relative to Hb C synthesis, intracellular modulation of a homogeneous population of precursor cells would predict a single-mean value for Hb C concentration when individual cells are measured. This was not observed. Approximately 45% of the cells did not react with anti-Hb C antiserum. The remaining reacting cells contained variable amounts of Hb C. These results indicated that selective mitogenesis of early precursor cells may be occurring. These results are highly tenative. Control experiments are in progress to establish the antiserum threshold sensitivity, the extent of variation of total hemoglobin in reticulocyte populations, and the actual temporal diversity of the reticulocytes isolated. Considerable improvement in the quantitation of micro-immunoprecipitates is also necessary if sub-populations are to be resolved on the basis of mean Hb C contents.

3) Shortly before birth the synthesis of fetal globin is replaced by the synthesis of adult β^A globin. Low levels of β^C globin are synthesized transiently during early neonatal life, or in response to anemic stress. In order to determine if expression of the β^A gene is required for the expression of the β^C gene, mid-gestation fetal marrow or liver was cultured at high concentrations of ESF. The synthesis of β^C globin was activated and little or no β^A globin was detected. Attempts to produce similar results in utero failed. Surgical bleeding or injection of ESF in utero did not activate β^C globin synthesis until shortly before birth, and then in only two of six fetuses. An allelic variation of the β^A gene is the β^B gene, which when expressed does not switch to β^C globin synthesis. Hybridization experiments demonstrated that sheep homozygous for β^B do not have the β^C gene. Colonies grown from cells of fetuses heterozygous for β^A and β^B failed to express either β^A or β^B globin,

but did respond to high ESF concentrations by synthesizing intermediate levels of β^C globin. The β^C gene appears to be independently activated by ESF. The failure of β^C activation in utero strongly suggests that other factors are present in the uterine environment which are able to antagonize the expression of B^C globin genes while maintaining fetal globin synthesis. Presumably these factors are lost in the transfer of tissue from fetuses to the plasma clot cultures and are not present in the present supplements used in these cultures.

Significance to Biomedical Research and the Program of the Institute: Several human anemias (e.g. β-thalassemia and sickle cell) are characterized by normal α globin synthesis and abnormal β globin synthesis or function. A common feature of these anemias is that synthesis of fetal hemoglobins is normal. Human hereditary persistence of fetal hemoglobin synthesis is a mild anemia and requires minimal clinical management. A rational approach to treatment of anemia involving defects in β globin synthesis is therefore the therapeutic re-utilization of the fetal gene in adult life. The possibility exists that such re-utilization can be achieved by hormone therapy. Attempts to describe the basic cellular biology and hormone regulation of erythropoiesis in sheep are done with the goal of realizing this possibility.

Proposed Course of Experiments: The present model of erythroid development in plasma clot cultures cannot, in detail, be substantiated by current methods. Time-lapse cinematographic measurements are planned to confirm cell division number, time, and relationship to burst and colony morphology. We are now attempting to prepare specific anti-Hb C flourescent antiserum. With such antiserum, the point in the erythroid series where hemoglobin phenotype is modulated by ESF can be determined. Which colonies are stimulated by ESF to synthesize Hb C can be shown by immunofluorescent microscopy. Those colonies will then be identified by burst morpholoy and projected area measurements and the point in the erythroid series where ESF acts thus established. Additional attempts to enhance the quantitative accuracy of single reticulocyte hemoglobin measurements by micro-immunoprecipitation will be made. Efforts will center around computer-assisted video and statistical methods. Severe anemia, i.e., elevated ESF levels, does not usually lead to increased fetal hemoglobin synthesis in man. However, recent experiments (Stammatoyannopoulos, et. al., Blood 48, 981) suggest that ESF may stimulate Hb F in vitro. These observations are analagous to the in vitro Hb C induction seen in fetal sheep erythroid tissue. The Hb F to Hb C switch in fetal sheep is therefore an excellent experimental model to define additional hormonal or microenvironmental factors which specify hemoglobin phenotype. Such experiments will be initiated.

Publications:

Barker, J. E., Pierce, J. E., Kefauver, B. C., and Nienhuis, A. W. Hemoglobin switching in sheep and goats: IX. Induction of hemoglobin C synthesis in cultures of sheep fetal erythroid cells. Proc. Nat. Acad. Sci., USA. In Press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02206-04 CHB

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Regulation of the Sheep Globin Genes

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

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	A.W. Nienhuis	Branch Chief	CHB,NHLBI
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	J.A. Kantor	Visiting Expert	CHB,NHLBI
	J.B. Pierce	Chief, Ungulate Section, NIH Animal Center	
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COOPERATING UNITS (if any)

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NIH Ungulate Unit
NIH Animal Center

LAB/BRANCH

Clinical Hematology Branch

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NHLBI, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The purpose of this project is to define the mechanism of regulation of the globin genes in erythroid cells. Control of globin mRNA metabolism is being studied by molecular hybridization analysis. Purified complementary DNAs specific for the individual globin mRNAs (beta for adult hemoglobin and gamma for fetal hemoglobin are used to quantitate changes in the individual mRNAs during both normal ontogeny and experimental stress erythropoiesis. The molecular mechanism of erythropoietin is being analyzed by measurement of changes in β^A and β^G mRNAs during the erythropoietin induced switch from Hb A ($\alpha_2\beta_2^A$) to Hb C ($\alpha_2\beta_2^G$). Analysis of possible regulation at the DNA sequence level is being attempted by molecular cloning of the sheep globin genes.

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Objective: The objective of this project is to determine the normal mechanisms regulating the expression of globin genes in erythroid cells. There are several different levels at which regulation might occur, e.g., gene transcription, nuclear RNA processing, globin messenger RNA translation, or alteration of globin mRNA stability. The globin genes of sheep serve as a suitable model for investigating these phenomena since the several individual globin genes are expressed only under specific conditions. Thus, the gamma globin gene (Hb F: $\alpha_2\gamma_2$) is expressed during fetal life, while the allelic adult beta globin genes (β^A and β^B) are expressed in adult animals making the normal adult hemoglobins, Hb A ($\alpha_2\beta_2^A$) and Hb B ($\alpha_2\beta_2^B$). The β^C globin gene (Hb C) is expressed transiently in newborn animals and in adults made anemic. Induction of β^C globin synthesis is specifically stimulated by erythropoietin both in vivo and in vitro. This is the only unique globin gene known to be specifically responsive to this erythropoietic hormone.

Extensive evidence exists which indicates that synthesis of these globins is not regulated at the translational level. Thus, our efforts are currently focused on study of mRNA transcription, processing, and stability in an effort to determine the role of each in regulating the amount of individual globin mRNAs, especially the β^C globin mRNA, since this serves as a specific molecular marker for the action of erythropoietin on early erythroid cells.

Methods: Reticulocyte-rich blood, bone marrow erythroid cells, and fetal liver erythroblasts are obtained from sheep synthesizing Hb A, Hb B, Hb C, or Hb F. Globin messenger RNA is purified, and radioactive DNA copies (cDNAs) are prepared by incubation with the enzyme reverse transcriptase. Each of the mRNA and cDNA preparations contain roughly equal amounts of sequences specific for alpha globin and sequences specific for the particular non-alpha globin produced in the erythroid cells of the animal being studied. In order to purify the individual alpha and non-alpha cDNAs, partially mismatched (heterologous) hybrids are formed between one type of mRNA (e.g., Hb A mRNA: $\alpha + \beta^A$ mRNA) and a heterologous cDNA (e.g., Hb C cDNA: $\alpha + \beta^C$ cDNA) under permissive conditions of hybridization. The hybrids are then incubated at a temperature known from preliminary experiments to denature only the partially mismatched hybrids (e.g. β^C cDNA-β^A mRNA) leaving the perfectly matched (alpha cDNA-alpha mRNA) hybrids intact. The single stranded (e.g., β^C cDNA) and double stranded hybrids (alpha mRNA-alpha cDNA hybrids) are then separated by hydroxylapatite chromatography. Purified cDNAs specifically complementary to α, β^A, β^B, β^C, and γ mRNAs are obtained in this way.

Each cDNA is used as a molecular hybridization probe to quantitate the amounts of the individual complementary mRNA in erythroid cells during ontogeny, and during the course of Hb C induction following various methods of erythropoietin treatment. A hybridization system has been developed to permit complete specificity of hybridization (vide infra). The amounts of mRNAs in these cells is compared to other cellular parameters such as globin synthesis, capacity for erythroid colony synthesis formation, and commitment of early erythroid progenitor cells to form colonies synthesizing Hb C.

In order to prepare synthetic globin DNA containing the double stranded base sequence of the structural globin genes, cDNA is incubated with DNA polymerase I, or with reverse transcriptase in a modified reaction, to syn-

thesize in vitro the complementary second strand of the gene. These genes are being prepared for molecular cloning, using recombinant DNA methodology.

Major Findings:

1. The individual α, β, and γ cDNAs have been purified using the above procedure. This procedure was developed on the basis of observations about the close nucleotide base sequence homology of sheep globin mRNAs made in this laboratory. Studies of the thermal stability of heterologous hybrids (e.g., β^A cDNA-β^C mRNA) and homologous hybrids (e.g., β^A cDNA-β^A mRNA) showed that the heterologous duplexes had a T_m only 2.5-5°C below the T_m of perfectly matched duplexes. Thus, the differences in nucleotide base sequences among the various beta and gamma mRNAs were near the minimum expected on the basis of the number of amino acid sequence differences among their globin chain products. Because of this close sequence homology, previously available methods for preparing pure cDNAs were not suitable and the above procedure, using selective thermal denaturation, was developed. The sheep globin genes are unique among mammalian globin genes in the degree to which the nucleotide base sequences have been conserved.

2. A molecular hybridization assay permitting specific quantitation of each globin mRNA with the purified cDNAs has been developed. Because of the extreme degree of sequence homology, it was necessary to conduct the hybridization assays at incubation temperatures only 2-3°C below the T_m of perfectly matched duplexes. Indeed, heterologous β^A-β^B cDNA-mRNA duplexes have T_m's only 0.8-1.0°C below the T_m of the corresponding homologous duplexes, and could not be distinguished in any hybridization assay employed. However, all other combinations of cDNA and mRNA could be specifically quantitated. Incubation of annealing mixtures at temperatures approaching the T_m were found to have specific and predictable effects on the rate and extent of hybridization. Since the β^A-β^B heterologous duplexes could not be distinguished from homologous duplexes, the development of this hybridization assay permitted partial definition of the limits of specificity of liquid molecular hybridization assay systems.

3. Using the specific molecular hybridization assays, it was shown that the switch from Hb F to Hb A during the neonatal period, and the switch from Hb A to Hb C during anemia are mediated by corresponding changes in the actual chemical amounts of the γ, β^A, and β^C mRNAs. No evidence for translational control was obtained. The kinetics of the mRNA conversion were consistent with the hypothesis that the switch in the patterns of gene expression occurred in early erythroid cells prior to the onset of morphologically recognizable differentiation.

4. DNA-cDNA hybridization conditions permitting measurement of the individual globin genes in cellular DNA were also developed. The inability of sheep homozygous for Hb B to switch to Hb C was shown to be due to absence of the β^C gene from the genome of these animals.

5. The γ and β^A globin genes were shown to be present in the DNA of erythroid cells synthesizing only Hb C. Thus, selective deletion of the non-expressed globin genes in erythroid cells was eliminated as a possible mechanism

of regulation. In parallel experiments, the non-expressed globin genes were also shown to be retained in erythroid cells of humans synthesizing only Hb A.

6. β^C globin mRNA was shown to accumulate in early erythroid cells only 24-48 hrs after erythropoietin exposure, even though commitment of early erythroid cells to give rise to colonies producing Hb C occurs within the first 6-12 hrs after exposure. No preferential accumulation of β^C globin mRNA sequences was detected in the nuclei of early or late erythroid cells. Thus, β^C globin mRNA accumulation appears to be a relatively late effect of erythropoietin on the cells. Moreover, selective intranuclear processing or stability of β^A or β^C globin mRNA sequences transcribed at equal rates does not appear to be the sole mechanism of regulation.

7. Double stranded DNA containing the structural gene sequence of each of the sheep globin genes has been prepared. Preliminary analyses of this material suggests that it is in a chemical form suitable for insertion into bacterial plasmids followed by molecular cloning and subsequent amplification of the recombinant plasmids. Preparation of sheep globin gene sequences in this way would permit the purification of large amounts of 100% pure gene sequences for use as hybridization probes and affinity column ligands. This material will be used to isolate the native globin genes and surrounding regulatory sequences, to establish the base sequence of the various genes, to identify and characterize the nuclear mRNA precursors, and to develop specific hybridization analyses more sensitive and precise than currently available.

Significance to Biomedical Research and the Program of the Institute: The globin genes, especially of the sheep, provide a unique model for investigating gene regulation in eukaryotic cells. The β^C globin gene of sheep also provides a specific "marker" for the molecular effects of erythropoietin on erythroid cells. Knowledge gained by study of these globin genes will be applied to the study of human disease states, specifically thalassemia, and also to the regulation of human globin genes.

Proposed Course of the Project: The base sequences, mRNA metabolism, and kinetics of erythropoietin response, of the sheep globin genes and their mRNAs will be further characterized utilizing the superior methods permitted by the availability of sheep globin DNA cloned in *E. coli*. Classes of non-globin mRNA are being prepared which respond specifically to the action of erythropoietin. The globin genes will be purified from cellular DNA in an effort to characterize adjacent regulatory sequences. Finally, isolation and characterization of the nuclear mRNA precursors of each globin mRNA is being attempted. Knowledge gained from the structure and kinetics of induction of these sequences will be used to determine the mechanism by which individual globin genes are selected for expression in primitive erythroid cell progenitors.

Publications:

1. Benz, E.J., Jr., Geist, C., Steggles, A.W., Barker, J.E., and Nienhuis, A.W.: Hemoglobin switching in sheep and goats: VII. Preparation of complementary DNA's specific for the alpha, beta, and gamma globin messenger RNA's of sheep. J. Biol. Chem. 252: 1908-1977.

2. Benz, E., Jr., Turner, P.H., Barker, J.E., Nienhuis, A.W.: Stability of the individual globin genes during erythroid differentiation. Science 196: 1213-1214.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 02207-04 CHB

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Globin Gene Expression in Somatic Cell Hybrids

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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Department of Biology, Yale University

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

It is the purpose of this project to determine the chromosomal localization of the human globin genes and to analyze the regulation of globin gene expression in somatic cell hybrids. To determine the chromosomal assignments, hybrids between mouse and human fibroblasts are analyzed to obtain a precise estimate of chromosomal composition. DNA extracted from these cells is analyzed to determine the human globin gene content. These studies have resulted in the assignment of the human alpha globin gene to chromosome 16 and the human beta globin gene to chromosome 11. Hybrids between mouse Friend erythroleukemia cells and human cells are analyzed to determine the mechanism of regulation of the human globin genes. Co-expression of human and mouse globin genes has been obtained and further hybrids are under analysis to determine whether both the human beta (adult hemoglobin) and gamma (fetal hemoglobin) are expressed in these hybrid cells.

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Objectives:

The goal of the work during the past 15 months have been focused on obtaining the chromosomal assignments of the globin genes. To this end, many hybrid cells have been analyzed, both as to chromosome composition and human gene content. Now that the chromosome assignment and the linkage patterns of the human globin genes are known, the second objective of this project may be more readily achieved. Thus the presence or absence of human alpha and beta genes in particular hybrids between MEL cells and human cells can be determined by simple isozymal analysis and the expression of these genes can be correlated with various properties of the hybrid cells.

Methods:

1. Analysis of hybrid cells for globin genes: Cells are expanded to provide 2-5 x 10^9 cells for DNA extraction. DNA is recovered by phenol chloroform extraction, ribonuclease digestion, and ethanol precipitation. DNA samples sonicated to a standard fragment size (300-500 base pairs) are annealed to purified human alpha and beta globin complementary DNA. These cDNAs are prepared by reacting reverse transcriptase with human globin mRNA preparations enriched in alpha or beta mRNA sequences respectively. The annealing reactions are performed under sufficient stringency to prevent reaction of the human globin cDNAs with mouse globin genes present in the hybrid cells.

2. Chromosomal composition of the hybrid cells: Human and mouse chromosomes are distinguished by Hoechst's fluorescent centromeric stain. Giemsa trypsin banding is then performed on the same metaphase spreads to identify the particular human chromosomes. Human and mouse chromosomes are also distinguished by Giemsa 11 staining and translocations are identified by this technique. Furthermore human isozymes represented in particular hybrid cells are determined by appropriate analysis.

3. Somatic cell hybrids are generated by Sendai virus mediated fusion between mouse erythroleukemia cells and human erythroid, lymphoid, and fibroblast cells. The presence or absence of the human alpha and beta globin genes are determined by isozymal analysis of extracts from these cells. Lactic dehydrogenase A (LDH-A) is known to be linked to the human beta gene and adenosyl phosphoriboseal transferase (APRT) is known to be linked to the alpha gene providing convenient markers to establish the presence of these genes in particular hybrid cell clones. Cells are then induced to make hemoglobin by exposure to dimethylsulfoxide and RNA is extracted. The presence of human globin mRNA sequences is determined by annealing to appropriate purified complementary DNA.

Major Findings:

1. Analysis of 16 separate hybrid clones has yielded the observation that the human alpha globin gene is localized to chromosome 16. The presence of a selectable marker on this chromosome (APRT) and the availability of a number of hybrid lines in which the mouse APRT is absent allowed us to definitively confirm this assignment by selection experiments. An excellent correlation was established in these selection experiments between the presence of chromosome 16, the isozymal activity of human APRT, and the presence and quantity of human alpha globin gene sequences.

2. The human beta globin gene has been shown to be on chromosome 11 by analysis of an additional ten hybrid lines. An excellent correlation between a marker for 11, LDH-A, and the human beta gene has been established. Furthermore, the chromosomal composition of these clones has confirmed that chromosome 11 is present when the human beta gene is found.

Significance to Biomedical Research and Institute Program:

An understanding of the mechanism of mammalian gene regulation is of fundamental importance in understanding human genetic disease. This program offers an opportunity for insight into genetic regulation by exploring our knowledge of the expression of the globin genes in somatic cell hybrids. Now that the chromosomal assignments of the human globin genes have been established, further analysis of their linkage relationships may permit exploitation of this knowledge in prenatal diagnosis of hemoglobinopathies.

Proposed Course of this Project:

The analysis of co-expression of the individual linked human globin genes (gamma and beta) will be determined in appropriate somatic cell hybrids.

Publications:

1. Anderson, W.F., Deisseroth, A.B., Velez, R., Nienhuis, A.W., Ruddle, F.H., and Kucherlapati, R.S.: A new technique for mapping the human hemoglobin genes. In McKusick, V.A. (Ed.): Human Gene Mapping 3. National Foundation March of Dimes, 1976, pp 367-372.

2. Deisseroth, A., Velez, R., Burke, R., Minna, J., Anderson, W.F., and Nienhuis, A.: Extinction of globin gene expression in human fribroblast X mouse erythroleukemia cell hybrids. Somatic Cell Genetics 2: 373-384, 1976.

3. Deisseroth, A., and Nienhuis, A.: Study of markers of erythroid differentiation in somatic cell hybrids. In Vitro 12: 734-742, 1976.

4. Deisseroth, A., Nienhuis, A.W., Turner, P., Velez, R., Anderson, W.F., Ruddle, F., Lawrence, J., Creagan, R., and Kucherlapati, R.: Localizaton of the human alpha globin structural gene to chromosome 16 in somatic cell hybrids by molecular hybridization assay. Cell 1977, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02208-03 CHB

PERIOD COVERED
July 1, 1976 through September 30, 1977

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
Other:	W.L. Henry, Senior Investigator	CB	NHLBI
	D.T. Peterson, Clinical Associate	CHB	NHLBI
	E.J. Benz, Jr., Research Associate	CHB	NHLBI
	Jeffrey Borer, Senior Investigator	CB	NHLBI
	Patricia Griffith, Clinical Nurse Specialist	CHB	NHLBI

COOPERATING UNITS (if any)

Cardiology Banch, NHLBI

LAB/BRANCH
Clinical Hematology Branch

SE

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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)

These studies are designed to evaluate the clinical benefits achieved by iron chelation in patients with chronic iron overload. Detailed assessment of cardiac, endocrine, and hepatic function are performed prior to initiation of chelation therapy and these analyses are repeated periodically during the course of iron removal. Desferrioxamine is administered either intramuscularly or subcutaneously and the amount of iron removed is determined by accurate measurement of urinary iron excretion and careful recording of total iron administered by transfusion.

PHS-6040

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.

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 or iron removal by phlebotomy.

Clinical evaluation of organ function include the following:

1) Heart: 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 as assessed by resting ejection fraction. In addition, the configuration of the left ventricle and resting ejection fraction is determined by radionuclide cineangiography. Supine exercise is performed to 70% of maximal predicted heart rate and the ejection fraction is again determined by radionuclide cineangiography. By these methods a detailed analysis of cardiac structure and function is achieved.

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:

1. Retrospective analysis of our experience in treating 11 patients with a combination of intramuscular desferrioxamine and oral ascorbic acid has led to the conclusion that the latter agent may depress cardiac function. Thus 8 of the 11 had documented cardiac deterioration as determined by echocardiogram (reduction in ejection fraction). Of these, 6 had further clinical evidence of cardiac malfunction. Withdrawal of ascorbic acid was attended by return toward normal cardiac function over a period of 6-12 months in 5 of the 8 patients.

2. The pituitary gonadal axis was identified as being the most sensitive to the toxic effects of iron. Thus approximately 80% of the total patients evaluated (25) exhibited reduced gonadotropin production by the pituitary. Certain others had evidence of end organ (testes or ovary) failure as well.

3. Growth hormone production was found to be normal in all but one of our patients with iron overload. However, several patients had lowered somatomedin values, suggesting this is a possible mechanism for growth failure in the chronically transfused iron overloaded patient with congenital hemolytic anemia.

4. Chronic subcutaneous administration of desferrioxamine for 12-22 hours each day was found to provide iron removal. Most patients over the age of 10 achieved iron balance by this regimen and certain others excreted sufficient iron so that the amount administered by transfusion was exceeded substantially.

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:

The project will be continued until a suitable iron chelator is found and evaluated or until the need for transfusion in thalassemia and other congenital hemolytic anemias is removed. Currently we are accumulating a series of 30-35 patients of various ages who are treated intensively with chronic subcutaneous desferrioxamine. Since the natural history of this disorder is well established, we will attempt to determine whether any deviation in the incidence of caridac, endocrine, and hepatic complications ensues from this therapeutic effort. Additional trials will include the use of controls to test specific aspects of treatment, e.g., the use of low dose ascorbic acid to enhance iron excretion achieved by subcutaneous desferrioxamine.

Publications:

1. Nienhuis, A.W., Delea, C.A., Aamodt, R., Bartter, F.C., and Anderson, W.F.: Evaluation of desferrioxamine and ascorbic acid for the treatment of chronic iron overload. National Science Foundation Birth Defect Series, 12: 177-185, 1976.

2. Nienhuis, A.W., and Anderson, W.F.: Treatment of transfusional hemosiderosis with desferrioxamine and ascorbic acid. Symposium on the Development of Iron Chelators for Clinical Use. Anderson, W.F. and Hiller, M.C. (Eds.). Development of Iron Chelators for Clinical Use, U.S. Department of Health, Education, and Welfare, Publication No. (NIH) 76-994-1976, pp. 115-122.

3. Propper, R.D., Rosenthal, A., Cooper, B., Rufo, R.R., Bunn, H.F., Nienhuis, A.W., Anderson, W.F., and Nathan, D.G.: Continuous subcutaneous administration of desferrioxamine in patients with iron overload. New Engl. J. Med., 1977, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02209-04 CHB

PERIOD COVERED

July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Cardiac Hemolytic Anemia

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

PI:	D. M. Miller	Clinical Associate	CHB NHLBI
OTHER:	A. W. Nienhuis	Branch Chief	CHB NHLBI
	C. L. McIntosh	Senior Surgeon	CS NHLBI

COOPERATING UNITS (if any)

Cardiac Surgery Branch, NHLBI

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.25	.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)

This project investigates the etiologies of cardiac hemolytic anemia and possible medical therapies of this post-surgical complication. A group of patients has been characterized on the basis of hemolysis rate, valve function, ferrokinetics, and bone marrow iron stores. Selected patients are now being treated with a trial course of propranolol in an effort to decrease hemolysis rate, allowing the bone marrow to maintain the red cell mass at a more normal level.

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Objectives:

1) To characterize the factors giving rise to uncompensated cardiac hemolysis; and 2) to evaluate the effect of propranolol in altering hemolysis via alterations in heart rate and cardiac stroke velocity.

Methods: Patients are admitted for an initial period of evaluation during which parameters of hemolysis are measured and prosthetic valve function is evaluated. If they are judged suitable for the propranolol trial (i.e., no recent history of overt congestive heart failure and a relatively short red cell survival), the hematocrit is brought to 30-40% by transfusion. Red cell survival and other parameters of hemolysis are measured while the patient is on a constant exercise regimen. After two weeks the patients are again transfused to normal, and propranolol therapy is instituted in doses sufficient to lower exercise stimulated heart rate 15-20%. A chromium RBC survival is repeated and the hemolytic rate before and after propranolol therapy are compared.

Major Findings:

1. Three patients have been studied on propranolol; each has shown a modest decrease in hemolytic rate.

2. Two patients evaluated for propranolol treatment had prosthetic valve dysfunction, one with prosthetic mitral stenosis and the other with a perivalvular leak. These patients were referred for cardiac surgery.

Significance to Biomedical Research and the Program of the Institute: Cardiac hemolysis is a major postoperative complication of cardiac valve replacement. Many of those patients suffering this complication are poor surgical candidates. A medical therapy would avoid the need to subject these patients to the relatively high mortality of re-operation.

Proposed Course of the Project: Additional patients are being sought to study the effect of propranolol on cardiac hemolysis. If there is a significant effect in several of these patients without undue complications, they will be placed on long term propranolol. Those patients previously found to have inadequate bone marrow response to cardiac hemolysis will be re-evaluated periodically to determine factors responsible for the anemia.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02210-03 CHB

PERIOD COVERED

July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Regulation of the Respiratory Function of Blood

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

PI:	R. M. Winslow	Senior Investigator	CHB NHLBI
OTHER:	M. L. Swenberg	Research Chemist	CHB NHLBI
	R. Berger	Senior Investigator	LTD NHLBI
	M. Samaja	Visiting Scientist	CHB NHLBI
	R. Shrager	Mathematician	LSMM DCRT
	L. Rossi-Bernardi	Prof. Enzymology, Univ. Milan, Milan, Italy	
	S. Charache	Assoc. Prof. Med., Johns Hopkins Univ. Balto, Md.	

COOPERATING UNITS (if any) Laboratory of Technical Development, NHLBI
Laboratory of Statistical and Mathematical Methodology, DCRT
Biomedical Engineering and Instrumentation Branch, DRS
University of Milan. Johns Hopkins University, Baltimore, Maryland

LAB/BRANCH
Clinical Hematology

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	

CHECK APPROPRIATE BOX(ES)

[X] (a) HUMAN SUBJECTS [ ] (b) HUMAN TISSUES [ ] (c) NEITHER

[ ] (a1) MINORS [ ] (a2) INTERVIEWS

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

The objective of this project is to understand the molecular and cellular regulation of hemoglobin function. In this work we analyze normal and and abnormal human hemoglobin and various animal hemoglobins utilizing newly developed methods for measuring the whole blood oxygen dissociation curve and established methods for studying the dissociation curves of purified hemoglobin solutions. The data obtained so far is of sufficient quality that theoretical and mathematical models of hemoglobin function can be tested.

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Methods:

The methods used in this project stem from the cooperative efforts of investigators in the Clinical Hematology Branch, Laboratory of Technical Development and the Biological Engineering and Instrumentation Branch at the NIH and at the University of Milan and Johns Hopkins University.

1. The automatic continuous oxygenation apparatus used for measuring the whole blood oxygen affinity curve has been developed further. The system is now completely automated using a microprocessor for control of the experiment, recording of the data, calculation of the data, display by an X-Y plotter and storage on a flexible disc memory unit. This apparatus allows us to perform a far greater workload of experiments and clinical service with fewer mistakes and less dependence on the NIH computer system. A faster CO_2 electrode is under development by the Laboratory of Technical Development.

2. An improved reaction cuvette is being developed by BEIB which does not use the hydrogen peroxide principle, but rather exchange of oxygen across a permeable membrane. This method will allow the determination of the oxygen dissociation curve in both ascending and descending fashion with particular applicability to the problem of measurement of oxygen affinity of sickle cell anemia blood and concentrated hemoglobin solutions.

3. Oxygen dissociation curves of purified hemoglobin solutions are studied using a spectrophotometric method of Imai. This method allows very precise determination of the extremes of dissociation curves, but it is limited to dilute solutions.

Findings:

1. A standard oxygen dissociation curve for normal human blood has been measured and published. These data are of very high precision and are amenable to analysis in terms of various mathematical models. This work is carried out in conjunction with Dr. Richard Schrager of DCRT.

2. The effect of 2,3-DPG and pH on the position and shape of the oxygen dissociation curve has been studied. Below a molar ratio of 1/1 (DPG/Hb) DPG has a strong effect on the position and shape of the dissociation curve. However, above that ratio, the principle effect of 2,3-DPG within the red cell is the alteration of pH. The strong effect of 2,3-DPG on the shape of the oxygen dissociation curve has been described for the first time. This effect is probably of major physiologic importance since the effect on the upper part of the dissociation curve is very strong. Previous methods which measured only p50 were not sensitive to this important effect.

3. We have demonstrated that HbC of the goat is very sensitive to CO_2. In the presence of CO_2 its dissociation curve is shifted to the right relative to that of goat Hb A. In the absence of CO_2 these dissociation curves are identical. This finding is particularly surprising in view of the fact that Hb C has lost one of the principle 2,3-DPG and CO_2 binding sites, the N-terminal amino group of the β chain. The unique effect of CO_2 of Hb C may be of adaptive advantage under conditions of anemia or high altitude.

4. Studies of the oxygen binding capability of several abnormal hemoglobins including Hb Providence, Hope and Potomac have been carried out in collaboration with Dr. Samuel Charache. The study of these mutants has indicated that reversal of the gradient of oxygen affinity between maternal and fetal blood does not lead to decreased fetal survival and that decreased whole blood oxygen affinity of a patient with hemoglobin Hope/β^0 thalassemia compensates for the moderate anemia seen in this individual.

Significance to Biomedical Research and the Program of the Institute: The delivery of oxygen to tissues is the primary respiratory function of blood. The present studies are a beginning of the quantification of the effect of various mediators of whole blood oxygen affinity. These studies can further our understanding of adaptive mechanisms during anemia, respiratory disease, high altitude, and abnormal hemoglobins which affect the ability of the red cell to deliver oxygen to tissues.

Proposed Course of the Project:

1. Continued evaluation of the effects of pH, 2,3-DPG and CO_2 on the whole blood oxygen equilibrium curves will be carried out chiefly in conjunction with the University of Milan.

2. The new method of studying the equilibrium curve in the ascending and descending fashion will be employed to study concentrated pure hemoglobin solutions. Such data will be compared with that obtained with whole blood and computer simulation of whole blood respiratory function will be continued.

3. A collaboration has been proposed with Professor Carlos Monge of the University of Lima, Lima, Peru, in the study of adaptation in high altitude inhabitants in the Andes. A controversy exists as to the exact erythrocytic adaptive mechanisms under such conditions and we believe that the newly developed methods which we have described can lead to a better understanding. High altitude adaptation and adaptation to anemia are probably very similar.

4. Further characterization of the oxygen binding properties of Hb A and C of the goat will be carried out. In addition to describing the effects of CO_2, 2,3-DPG, and pH on these hemoglobins characterization of the binding site of CO_2 on Hb C will be attempted using NMR techniques.

Publications:

1. Winslow, R.M., Swenberg, M.L,, Berger, R.L., Shrager, R.I., Luzzana, M., Samaja, M., and Rossi-Bernardi, L.: Oxygen equilibrium curve of normal human blood and its evaluation by Adair's equation. J. Biol. Chem. 252: 2331-2337, 1977.

2. Charache, S., Fox, J., McCurdy, P., Kazazian, H., Winslow, R.M.: Post Synthetic deamidation of hemoglobin providence. (B82 Lys Asm, Asp), and its effect on oxygen transport. J. Clin. Invest. 59: 652-658, 1977.

816

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02211-03 CHB

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Alteration of Blood Oxygen Affinity in the Treatment of Sickle Cell Anemia

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

P.I.:	R.M. Winslow	Senior Investigator	CHB NHLBI
Other:	D.M. Miller	Clinical Associate	CHB NHLBI
	M.L. Swenberg	Research Chemist	CHB NHLBI
	A.W. Nienhuis	Branch Chief	CHB NHLBI
	J. Fulmer	Senior Investigator	CHB NHLBI

COOPERATING UNITS (if any)

Pulmonary Branch, NHLBI
Laboratory of Technical Development, NHLBI

LAB/BRANCH

Clinical Hematology

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	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 objective of this work is to evaluate the effect of alteration of blood oxygen affinity in patients with sickle cell anemia. It is generally felt that an increase in oxygen affinity will lead to decreased sickling because a larger fraction of hemoglobin will be in the oxy-configuration at a given partial pressure of oxygen. However, diminished oxygen delivery to tissues might be a deleterious effect of increased oxygen affinity. We have developed reliable methods for measuring whole blood oxygen affinity in sickle cell anemia, and have learned that the position and shape of the oxygen dissociation curve of sickle blood is very dependent on the immediate history of the blood sample after venipuncture. Only under very special conditions is the measured value related to the in vivo oxygen transport capability of blood. The rate of sedimentation of SS red cells is exquisitely sensitive to O_2 tension. The value of this measurement in identification and evaluation of anti-sickling compounds is being explored.

9/7

PHS-6040

Methods:

1. A standard set of clinical studies is applied to a group of sickle cell anemia patients representing a broad spectrum of severity. The purpose of these studies is to characterize damage to specific organ systems in an attempt to correlate such dysfunction with various in vitro measurements of blood oxygen affinity.

2. Exercise testing using standard treadmill protocols is carried out in collaboration with the Pulmonary Branch.

3. Whole blood oxygen affinity is measured using methods which have been developed at the NIH in conjunction with the laboratory of Technical Development, NHLBI and the Biological Engineering and Instrumentation Branch.

4. A study of cell deformability in sickle cell disease as a function of PO_2 has been carried out using the technique of sedimentation of cells in the patients' native plasma. This method appears may be useful in evaluation of anti-sickling agents.

Findings:

1. The position and shape of the whole blood oxygen dissociation curve in sickle cell anemia is dominated by the effect of Hb S gelation and cell sickling. Many different types of experiments point to this conclusion and suggest that in vivo, it is very likely that Hb S agregation is never completely reversed, even when blood is oxygenated. The position of the curve is strongly dependent upon whether the blood is oxygenated or deoxygenated before the curve was determined; thus, blood whose dissociation curve is measured beginning at low pO_2 has markedly less affinity for oxygen than blood whose curve is measured beginning at high pO_2. This phenomenon is probably responsible for the wide discrepancy in the literature as to the actual position of the oxygen dissociation curve in sickle cell disease and probably also accounts for the poor correlation between blood oxygen affinity, irreversibly sickled cells, and clinical status in our patients. Since recognition of the partial reversibility of SS blood oxygenation, we have developed new methods for measuring in vivo oxygen affinity. Results with this method have not yet been applied to a sufficient number of patients to allow correlation with clinical status.

2. The degree of anemia and blood p50 are not closely correlated in approximately 50 sickle cell anemia patients. Furthermore, the number of irreversibly sickled cells present in the blood does not correlate with clinical status and only very poorly with whole blood oxygen affinity. These findings are not in agreement with previous studies reported in the literature and we believe they are explained by our findings in regard to measurement of whole blood oxygen affinity noted above. As yet, we have not found any clear cut property of sickle cell anemia blood which is an accurate predictor of clinical status.

3. Treadmill exercise studies have been carried out on 11 patients. These studies indicate severe exercise intolerance in most patients. Our initial measurements indicated a marked reduction in blood oxygen affinity in these patients which should theoretically have compensated for their anemia and allowed more normal exercise tolerance. Repeat analyses of blood oxygen affinity in these patients will be necessary using newer methods (see above).

4. The sedimentation rate of sickle cells in their own plasma was found to be markedly dependent upon the degree of oxygenation of the cells. Cells which are completely deoxygenated do not settle in their own plasma, whereas those which are completely oxygenated settle very rapidly. It is belived that the rate of settling is sensitive to cell shape which changes upon sickling. We have demonstrated that agents which are known to inhibit sickling, strongly influence the erythrocyte sedimentation rate and we therefore feel that this test has potential as a means for testing anti-sickling drugs. It may also be useful as a method for the quantitation of the sickling phenomenon under in vivo conditions in individual patients.

Significance to Biomedical Research and the Program of the Institute:

Sickle cell anemia is a chronic disease which produces much suffering and incapacity of its victim. Our studies are directed toward understanding the physiological basis for organ dysfunction in this disorder as it relates to oxygen transport capacity of blood and the sickling phenomenon. Our studies have suggested that Hb S aggregation may not be fully reversible under in vivo conditions. Furthermore, the best measurements of the oxygen affinity of SS blood indicate only a modest reduction in affinity compared to normal blood. Thus the anemia of sickle cell disease is not compensated by reduced oxygen affinity as previously had been thought. Further characterization of the oxygen affinity of SS blood may lead to the recognition of safe and effective anti-sickling agents which do not interfere with oxygen transport.

Proposed Course of the Project:

1. We will continue the evaluation of sickle cell anemia patients to attempt to discover factors which might account for differences in clinical severity. These patients will be candidates to receive various anti-sickling agents as they become available and undergo testing in our laboratory.

2. We will continue to refine the methods of exercise testing in an attempt to define, from a functional point of view, the respiratory function of blood.

3. We will begin an evaluation of a number of anti-sickling compounds using methods which have been developed by us (whole blood oxygen dissociation curves and the oxygen dependence of the erythrocyte sedimentation rate.)

4. We will continue to investigate the irreversibility of the oxygenation reaction within red cells and attempt to develop a model for the application of this information to in vivo conditions. We are currently working with LTD and BEIB to develop a method for the measurement of the oxygen equilibrium curve starting either at high or low pO_2. This method, which already has produced promising preliminary data will be useful in the study of the reversibility of the sickle cell blood oxygenation reaction.

5. In conjunction with the Laboratory of Technical Development, we will attempt to develop methods for studying the oxygenation of individual sickle erythrocytes.

6. We are planning to initiate studies to characterize hemoglobin degradation products (methemoglobin and hemichromes within sickle cells.

Publications:

1. Winslow, R.M., Blood oxygen equilibrium studies in sickle cell anemia. Proceedings of the Symposium on Molecular and Cellular Aspects of Sickle Cell Disease, U.S. Dept. of HEW, publication No. (NIH) 76-1007, 1976 pp 235-256.

2. Winslow, R.M., Swenberg, M.L., Samaja, M., Jackson, R.G., Nienhuis, A.W., The clinical significance of whole blood oxygen affinity in sickle cell anemia. Proceedings of the International Conference on Sickle Cell Disease: A World Health Problem. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02300-01 CHB

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Structure of the Globin Genes in Chromatin

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

PI.	Donald Miller	Clinical Associate	CHB NHLBI
	Neal Young	Visiting Expert	CHB NHLBI
Other:	Richard Croissant	Staff Fellow	CHB NHLBI
	Arthur W. Nienhuis	Branch Chief	CHB NHLBI
	David Axelrod	Research Biologist	MHB NHLBI

COOPERATING UNITS (if any)

Molecular Hematology Branch, NHLBI

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	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)

The goal of this work is to determine the structural organization of the individual globin genes in chromatin. The following questions are under direct investigation: 1) Are there detectable structural differences between expressed and non-expressed globin genes in chromatin from erythroid cells? 2) What are the changes in chromatin structure which accompany the developmental sequences characteristic of erythroid maturation in normal and transformed mouse Friend erythroleukemia cells in culture? and 3) What is the component in chromatin (e.g. non-histone protein, modified histone, etc.) which underlies the structural features of the individual globin genes? Currently pancreatic DNAse I is used as a probe of chromatin structure since transcriptionally active genes have been shown to be exquisitely sensitive to brief exposure of nuclei to this enzyme. In vitro transcription of isolated chromatin by RNA polymerase has also been used as a probe of the configuration of globin genes.

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Objectives:

Only a restricted portion of the total DNA sequences in individual differentiated cells are expressed. Primary regulation of these sequences is imposed by their individual structure in chromatin. Chromatin is composed of histones arranged in "nu bodies" which take the form of beads strung on the DNA double helix. Non-histone proteins are also found in chromatin and may be concentrated in the transcriptionally active fraction although they are also associated with nu bodies. The structural arrangement of DNA sequences in chromatin results in only 6-10% of individual genes being available for transcription in specific cells.

The goal of this project is to understand the structural features of chromatin which result in specific transcription of individual globin genes. We wish to learn whether modulation of expression of specific genes, e.g., the closely linked γ and β genes of human are regulated at the level of chromatin structure. Therefore we are utilizing available techniques to probe the gene configuration and to determine its transcribability. Ultimately we hope to identify those structural components which are directly responsible for modulation of chromatin structure and gene transcribability.

Methods:

1. Nuclei are prepared from various erythroid tissues using standard techniques. The tissues include bone marrow from animals in which anemia has been induced by phenylhydrazine. Fetal erythroid liver from sheep are utilized for certain experiments. Patients with various hemolytic anemias who have spontaneous erythroid hyperplasia are also utilized. In addition, certain experiments have employed the transformed mouse Friend erythroleukemia cell which can be maintained in tissue culture and induced to undergo erythroid maturation in response to specific stimuli.

2. For certain experiments fractionation of erythroid cells is necessary. This is accomplished by glycol hypaque isopynic density gradient centrifugation which results in a pellet of highly mature erythroid precursors and a fraction of earlier cells in supernatant. Either of these two fractions is further fractionated by unit gravity sedimentation using the "staput" apparatus. Highly purified fractions of early and late cells can be obtained by this two-step fractionation method allowing comparison of the configuration of the globin genes in these cell populations.

3. Nuclei are exposed to pancreatic DNAse I for a time sufficient to digest 5-15% of the total nuclear DNA. The residual DNA and also DNA from control nuclei (not exposed to pancreatic DNAse) are recovered after proteolytic removal of the chromatin proteins.

4. The globin gene content of the control and residual DNA from nuclei exposed to pancreatic DNAse are quantitated by hybridization analysis. Globin complementary DNAs (cDNA) of mouse, rabbit, sheep, and human are prepared by incubation of appropriate mRNAs with reverse transcriptase. cDNA excess hybridization analysis is performed, providing a sensitive measure of the quantity of globin genes in the individual DNA samples.

Major Findings:

1. Bone marrow is composed of a mixture of erythroid cells of varying degrees of maturity ranging from early proerythroblasts to terminal orthochromatophilic erythroblasts. We have found that only half of the globin genes (the globin genes in half of the cells) are destroyed by exposure of nuclei from unfractionated bone marrow to pancreatic DNAse. The globin genes in nuelei from early bone marrow cells (proerythroblasts and basophilic erythroblasts) exhibit minimal sensitivity to pancreatic DNAse despite the presence of substantial amounts of globin mRNA molecules in these cells. The globin genes in late orthochromatophilic erythroblasts are quite sensitive, indicating that there may be a progressive increase in panc DNAse sensitivity during erythroid maturation. We conclude that panc DNAse sensitivity is not uniformly associated with transcribability of the globin genes or that the globin genes in individual cells may be only intermittently sensitive to panc DNAse (possibly related to cell cycle).

2. Chromatin prepared by standard techniques (including exposure to 0.35 M NaCl and 20 mM EDTA) was analyzed by exposure to panc DNAse. The globin genes were completely insensitive to this enzyme although globin genes in chromatin prepared by less harsh techniques which avoided exposure to salt and EDTA were as sensitive as in nuclei. Thus panc DNAse sensitivity appears to provide an index of the structural integrity of chromatin which may be useful in approaching the *in vitro* transcription of these genes.

3. Chromatin prepared by standard techniques has been transcribed with *E. coli* RNA polymerase in the presence of mercuriated UTP. This results in production of a RNA which contains mercury atoms allowing this RNA to be isolated by sulfyhydral affinity chromatography. This technique is useful in separating globin mRNA sequences which contaminate the chromatin from those which are synthesized during the incubation period. Using this method we have found that globin mRNA sequences are very inconsistently transcribed from chromatin isolated from erythroid cells. These observations plus the knowledge that the structural integrity of chromatin may be destroyed by the isolation techniques (as revealed by the panc DNAse assay) has led us to abandon *in vitro* transcription at least temporarily until better methods for preparing structurally intact chromatin can be formulated.

4. We have studied the panc DNAse sensitivity of mouse globin genes in nuclei isolated from Friend erythroleukemia cells. Nuclei were prepared from uninduced cells and also those exposed to inducer (DMSO) for 5 days. The globin genes from the uninduced cells were completely sensitive to panc DNAse despite a very low level of transcription as reflected by the low globin mRNA content of these cells. There were no significant changes in panc DNA sensitivity with induction indicating that primary modulation of the accumulation of globin mRNA in these cells during the induction process is not imposed by the structural configuration of chromatin

5. Nuclei have been isolated from various erythroid tissues of sheep. The tissues were selected so that cells actively transcribing the γ, B^A, β^B,

or β^C genes were obtained. After exposure to panc DNAse the isolated DNA was analyzed for its individual globin gene content. A complication was encountered. Since the sequences of these genes are closely related it proved difficult to obtain specific quantitative hybridization using the cDNA excess technique. Therefore sufficient DNA is being isolated to allow hybridization under DNA excess conditions to highly purified and highly radioactive [^{32}P] cDNA. The goal will be to determine whether the structural basis for modulation of expression of these individual globin genes lies in chromatin.

6. Nuclei have been isolated from bone marrow cells in patients with hemolytic anemia. After panc DNAse digestion, the DNA is isolated and the individual content of human alpha and beta globin genes is determined. Preliminary results indicate that both the gamma and beta genes are sensitive to panc DNAse, suggesting that modulation of expression of these genes is not at the level of chromatin structure.

Significance to Biomedical Research and the Program of the Institute: These studies have allowed us to gain insight into chromatin structure in erythroid cells during normal maturation and during induction of transformed cells (mouse erythroleukemia cells). Ultimately we will be able to understand better the induction of globin mRNA synthesis at the molecular level and the mechanism of differential expression of the individual globin genes. Our desire is to understand the Hb F to Hb A switching in humans and to induce Hb F synthesis in those patients with severe hemoglobinopathies in which fetal hemoglobin might replace a deficient or malfunctioning adult hemoglobin molecule.

Proposed Course of the Project: We intend to resolve the issue of the structure of the individual globin genes in chromatin. An effort will be made to refine the preparation of chromatin to preserve the globin genes in the natural state. Furthermore, we hope to isolate and demonstrate by reconstitution experiments the structural component which results in the transcribability of the individual globin genes.

Publications:

1. Wilson, G.N., Steggles, A.W., Anderson, W.F., and Nienhuis, A.W.: Transcription of chromatin by eukaryotic and prokaryotic polymerases. Meth. Cell Biol. in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02301-01 CHB

PERIOD COVERED

July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Effect of Partial Exchange Transfusion on Oxygen Transport in Sickle Cell Anemia

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

PI:	R. M. Winslow	Senior Investigator	CHB NHLBI
OTHER:	H. Klein	Assistant Chief	BB CC
	N. J. Statham	BioLab Technician	CHB NHLBI
	A. Lewis	Biologist	BB CC

COOPERATING UNITS (if any)

Blood Bank, Clinical Center, NIH

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.6	.3	.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)

Partial exhange transfusion is used in sickle cell anemia when surgery is indicated, when life-threatening complications (for example bilobar pneumonia) occur, or when a patient is totally incapacitated from recurrent crises. Because stored blood has reduced 2,3-DPG (thus, high oxygen affinity) and since the oxygen affinity of sickle cell blood is low, infusion of large amounts of stored blood could cause dramatic alteration of the recipient's blood oxygen affinity. The present study has indicated that when frozen, washed, packed cells were used, oxygen affinity of the recipient's blood remained reduced for at least 24 hours after transfusion, even when a 75% exchange is achieved. We believe that this procedure significantly improves oxygen transport and reduces the risk of tissue hypoxia immediately after the transfusion.

825

Methods:

1. Whole blood oxygen affinity is measured according to the method of addition of H_2O_2 to deoxygenated blood which contains an excess of catalase. This method has been developed previously in our laboratory in cooperation with Laboratory of Technical Development, NHLBI and University of Milan.

2. Partial exchange transfusion is achieved (in adult, consenting patients with sickle cell anemia by the removal of 500 ml whole blood, infusion of 300 ml saline, removal of an additional 500 ml blood, then infusion of 4-5 units of frozen, washed, packed cells.

3. Standard Ashby immunologic techniques are used to separate recipient and donor red cells after transfusion to quantitate each fraction.

Major Findings:

Three patients have been studied. In each case, blood oxygen affinity of the recipient was high (p50, 42 mm). After partial exchange transfusion (50-75% transfused cells) a 24-48 hour "equilibrium" period was observed during which 2,3-DPG initially decreased, then returned to normal. p50 remained high then gradually decreased to normal (37 mm) after 24 hours.

This result is not that which would be expected, since the oxygen affinity of the donor blood should have normal functional properties: it's 2,3-DPG concentration is approximately normal. Although a full explanation is not yet at hand, we currently believe that intracellular pH of the donor cells is low (6.5-6.8) after the deglycerolization procedure, returning to normal (7.0-7.2) only after the 24-48 hr period after transfusion.

Significance of Biomedical Research and the Program of the Institute: The above findings indicate that considerable improvement in oxygen transport can be achieved in sickle cell anemia patients with the partial exchange transfusion technique, and the complicated manipulations of the blood such as rejuvenation of metabolites is not needed. Exchange transfusion appears to be an economic, safe, and simple method for protection of sickle cell anemia patients from elective stress such as surgery, and can improve blood oxygen transport during complications such as severe infections. Furthermore, when washed, frozen cells are used, patients on chronic transfusion regimens will not experience periods of reduced oxygen transport after transfusion.

Proposed Course of Project: Further studies will include partial exchange transfusion of additional sickle cell anemia patients, and study of other severely anemic patients who are transfusion dependent. Furthermore, in vitro studies will be carried out to determine the mechanism of reduced oxygen affinity in these subjects.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02302-01 CHB

PERIOD COVERED

July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Purification of Erythropoietin

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

PI:	N.S. Young	Visiting Expert	CHB NHLBI
OTHER:	A.W. Nienhuis	Branch Chief	CHB NHLBI
	A. Gallager	Chief, Hematology Division, John Cochran Veteran's Hospital, St. Louis, Missouri	

COOPERATING UNITS (if any)

Division of Hematology, Department of Medicine, John Cochran Veteran's Administration Hospital, St. Louis, Missouri

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.75	.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)

This study was initiated to prepare purified erythropoietin in quantities adequate for study of the biochemical and biological features of the molecule, particularly the binding of erythropoietin to early erythropoietic cells and its role in the initiation of specific genetic events. The methods employed have utilized known aspects of erythropoietin structure, especially its high carbohydrate content. Erythropoietin has been partially purified by binding to plant lectins liganded to Sepharose using affinity chromatography. The acidic nature of the erythropoietin molecule has permitted it to be separated from most other proteins by isoelectric focusing techniques. Small amounts of purified erythropoietin may be used to prepare antibodies of high specificity and affinity. These antibodies would be useful in purification of erythropoietin in bulk and in the development of a radioimmunoassay for ESF.

Methods and Significant Results: The starting material for our purification of ESF is commercially available crude sheep erythropoietin. This product is labeled by either iodination with the Hunter-Bolto ^{125}I reagent (specific activity 4 x 10^8 cpm/mg or methylation with ^{14}C formaldehyde (specific activity 5 x 10^6 cpm/mg. Erythropoietin activity is assayed using the exhypoxic mouse; this assay system is sensitive to 0.1 unit of ESF.

Wheat germ agglutin is bound to Sepharose by cyanogen bromide activiation. On application in phosphate buffered saline, approximately 98% of the protein in crude commercial erythropoietin fails to bind to this lectin and is eluted in the salt solution. The two percent of protein which is bound to the wheat germ agglutin may be eluted specifically with N-acetyl-D-glucosamine. One µ-mole of wheat germ agglutin binds 75 units of erythropoietin. Binding of ESF to wheat germ agglutin is variable among batches of commercial erythropoietin.

Erythropoietin has been subjected to isoelectric focusing on polyacrylamide gel. Functional activity is eluted from the pH range 2-4. The great bulk of protein, however, focuses to between pH 5-6. Large quantities of erythropoietin have been subjected to preparative isoelectric focusing in sucrose density gradients; up to 100 mg of protein may be applied using this method. Two peaks of activity have been observed. The first focuses at the pI of 3.5 and the second focuses at the cathodic end of the gradient, pH 5.5 - 7; the second peak is associated with the bulk of protein. We have interpreted the heterogenity of erythropoietin on isoelectric focusing to be due to variability in the number of sialic acid residues and, therefore the charge of the molecule. This variability in carbohydrate residues may also explain differences in binding of various erythropoietin preparations to wheat germ agglutin.

Proposed Course of Project and Significance to Biomedical Research: Employing these two methodologies, erythropoietin of high specific activity will be used as an antigen to prepare an antibody to the hormone. This antibody may then be employed in the preparation of large quantities of erythropoietin from both sheep and human sources, as well as in a radioimmunoassay for erythropoietin. A radioimmunoassay would have broad clinical application in investigating the mechanism of various anemias. Purified erythropoietin would be useful in many studies of erythropoietic development: for example, experiments would be designed to determine the site of binding of erythropoietin to erythropoietic cells, to investigate a cyclic AMP "second messenger" mechanism in developing erythroid cells, and to quantitate the effects of erythropoietin on well-defined genetic events in vitro, such as the β^A to β^C switch in the fetal lamb system.

Publications: None

ANNUAL REPORT OF THE
CLINIC OF SURGERY
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
July 1, 1976 through September 30, 1977

The clinical and laboratory programs of the Surgery Branch have, as in past years, largely centered upon the study of 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.

Aortic Valve Replacement in the Elderly: Encouraging Results. It has been generally considered that elderly patients with valvular heart disease would be poor operative candidates. In fact, there have been published opinions that in patients over 60 years of age operative treatment should not even be considered. The experience of the Surgery Branch is in marked contrast.

From 1966 through 1975, 73 patients age 60 years or more and 277 patients under age 60 underwent isolated aortic valve replacement. Eighty percent of all operative survivors underwent cardiac catheterization 5-9 months postoperatively. Followup has averaged six years per patient.

The hospital mortality for the elderly patients was 2.6%, compared to 5.7% in the younger group. The late mortality, due to heart disease, was 19% and 18%, respectively. There was a significant improvement ($p < .001$) in the LVEDP, cardiac index, and functional class of all patients in both the younger and older groups. For example, in the older patients undergoing operation for aortic stenosis, the LVEDP decreased from 20.4 to 14.9, the cardiac index increased from 2.4 to 2.8, and the average functional class improved from 2.7 to 1.4. The improvement was similar among the younger patients treated for aortic stenosis: thus, LVEDP 20.6 to 13.6, cardiac index 2.8 to 3.2, and functional class from 2.8 to 1.4. Similar hemodynamic improvement was found in both the older and younger patients who were operated upon for aortic regurgitation and combined aortic stenosis and aortic regurgitation.

The increasing age of the population will make cardiac operations more common in older patients. The findings of this study indicate that aortic valve replacement carries the same low risk and brings about similar symptomatic and hemodynamic improvement in patients older and younger than 60 years.

Assessment of the Mitral Orifice by Cross-Sectional Echocardiography Before and After Mitral Commissurotomy. It is a desirable goal to be able to evaluate precisely various cardiac malformations by noninvasive methods. For example, the severity of stenosis of the mitral valve has been studied for many years by standard M-mode echocardiography. Evaluation of this method has been shown not to be reliable in quantifying the severity of mitral stenosis. Cross-sectional (two dimensional) echocardiography was

utilized to make direct measurements of the mitral valve orifice in 10 patients who underwent mitral commissurotomy for rheumatic mitral stenosis. All patients were in functional class III or IV, and all had the typical physical findings of mitral stenosis. The diagnosis was confirmed preoperatively by right and left heart catheterization.

Mitral commissurotomy was carried out in all patients: in 9 by closed transventricular dilatation of the valve, and the 10th valve was opened under direct vision. The dimensions of the opened valve were estimated before and after commissurotomy by the surgeon. All patients survived operation and all were asymptomatic six months later. At that time they were again studied by cardiac catheterization and cross-sectional echocardiography.

Satisfactory two-D images of the mitral orifice were obtained pre- and postoperatively in all patients. Three images were measured in all patients, and in none did the three measured valve areas vary more than 0.3 $cm.^2$ The mitral orifice before and after operation was also estimated from the cardiac catheterization data applied to the Gorlin formula. The correlation between the two measurements was excellent -- the correlation coefficient being 0.91. In contrast the E-F slope, opening excursion of the anterior leaflet, and the distance between the two valve leaflets in early diastole all correlated poorly with both the cross-sectional area determined either from the echocardiogram or the catheterization data.

The study indicated that quantitative assessment of the severity of mitral stenosis by M-mode echocardiography is impossible. Cross-sectional echocardiographic assessments of the size of the mitral orifice, however, correlates well with intraoperative and hemodynamic assessments of the valve opening. It should prove a valuable noninvasive method for evaluating patients preoperatively and also for assessing the results of mitral commissurotomy.

An Attempt to Develop a Satisfactory Prosthesis to Replace the Trachea

A satisfactory device for replacement of resected portions of the trachea has never been developed. Replacement of the trachea is required when the organ is damaged by prolonged use of an endotracheal tube leading either to necrosis or the formation of permanent strictures. Dilatation, debridement, or local excision will occasionally be effective but cannot be applied when a significant length of trachea is damaged. Also squamous carcinoma, either primarily in the trachea or extending from a major bronchus, may only be treated by wide trachael resection.

An experimental study was conducted in dogs to evaluate porcine trachael segments preserved with glutaraldehyde. In 12 animals a segment of the trachea 4 cm. in length was resected and replaced with a trachael xenograft. After one week the first three animals died of asphixiation when the lining epithelium of the graft sloughed and occluded the airway. In subsequent animals the epithelium was removed before the xenograft was implanted. The remaining nine animals did well the first week after operation. After this time rapid and progressive stricture formation occurred at both anastomoses, and was documented by serial bronchoscopic and x-ray

examinations. The proximal anastomosis seemed to narrow more rapidly than the distal one, and the anterior half of each anastomosis narrowed more than the posterior half. By four weeks all animals demonstrated marked stridor and respiratory distress and were killed. Pathologic examinations showed that none of the xenografts was infected. All were encased in a capsule of dense fibrous tissue and none of the anastomoses evidenced leakage or disruption. On histologic study all grafts showed striking foreign body reaction and there was focal necrosis on all layers. Every anastomosis was narrowed 60% or greater and there was marked inflammation of the adjacent host trachea. It was concluded that the xenografts had not been preserved adequately by glutaraldehyde. Currently Hancock Laboratories, who supplied the xenografts used in these preliminary studies, are attempting to improve preservation techniques. The epithelium will be removed from the graft before treatment and the graft will be constructed in such a manner as to be completely surrounded by cartilaginous rings. These new grafts will be evaluated in additional animals.

Measurement of Intracardiac Shunts by Radioangiocardiography. Quantitative radionuclide angiocardiography (QRAC) has been utilized in patients to estimate the magnitude of left-to-right shunts, but the accuracy and reliability of the method have never been documented in an experimental model. Such was the purpose of studies this past year.

Atrial septal defects were created in 11 dogs during a brief period of inflow occlusion. A portion of the interatrial septum was excised after the venae cavae were occluded and the heart was put into ventricular fibrillation. The procedure required interruption of the circulation for less than one minute. The animals were allowed to recover for two weeks.

A baseline (normal) QRAC was performed in each animal prior to operation. This, and all subsequent radionuclide studies, were carried out with the animal lying supine, lightly anesthetized, and with the gamma camera positioned over the chest. A three to four mCi bolus of ^{99m}Tc-pertechnetate was injected into the jugular vein and rapidly flushed with saline. A pulmonary time-activity curve was generated from the transit of the bolus through the lungs. All QRAC's were analyzed using an algorithm which was a modification of the gamma function method of Maltz and Trevis.

The accuracy of QRAC in study of shunts was examined two ways. Each dog had 10 QRAC studies on different days over a three week period. Five dogs had four sequential QRAC's within a 90 minute period. Accuracy was also measured in two ways. Each dog had three cardiac catheterizations and shunt measurement by oximetry. In addition, 7 dogs were operated upon and pulmonary and systemic flows measured directly by electromagnetic flow probes placed around the aorta and the pulmonary artery. All animals were then killed and their hearts examined.

Every dog was proved to have an atrial septal defect. The reproducibility studies showed that day-to-day variability in the magnitude of the shunt ranged from 8 to 14%. The variation among repeated studies on the same day was 6 to 7% of the measured shunt. The oximetric data in 6 dogs

showed a coefficient of variation of 9%, compared to 10% for the QRAC measurements. There was excellent correlation between the pulmonary and systemic flows measured by the electromagnetic flowmeters and QRAC determinations. The correlation coefficient was 0.97. These experimental studies provide a basis for believing that QRAC is an accurate and reproducible method for estimating the magnitude of left-to-right shunts under a variety of conditions.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02623-17 SU

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Operative Treatment of Patients with Obstructive Cardiomyopathy

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

PI: Andrew G. Morrow, M. D., Chief, Clinic of Surgery, NHLBI

OTHER: Stephen E. Epstein, M. D., Chief, Cardiology Branch, NHLBI
Walter H. Merrill, M. D., Clinical Associate, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

Cardiology Branch

LAB/BRANCH
Surgery

SECTION
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INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2½	2	½

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)

Since 1960, a major interest of the Clinic of Surgery, NHLBI, has been the development of an operative method to relieve obstruction to left ventricular outflow in patients with idiopathic hypertrophic subaortic stenosis (IHSS). As of May 1, 1977, 173 patients have been operated upon in this Clinic. At operation, a wedge of left ventricular septum is resected. The operative mortality has been low, as has the incidence of major operative complications. One hundred thirty-eight patients have undergone detailed postoperative evaluation, including cardiac catheterization. There has been distinct symptomatic improvement in all survivors, and catheterization has confirmed partial or complete relief of obstruction in almost all patients. Long-term followup has not demonstrated recurrence of obstruction, and most patients continue to derive gratifying symptomatic and hemodynamic benefit. This experience demonstrates that wedge resection of the left ventricular septum in symptomatic IHSS patients with documented left ventricular outflow obstruction may be undertaken at low risk, and it provides long-term symptomatic and hemodynamic improvement.

833

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02624-02 SU

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Shunt Determination by Quantitative Radioangiocardiography in Experimental ASD

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

PI:
Donald C. Watson, M. D., Clinical Associate, Clinic of Surgery, NHLBI

OTHER:
Vincent A. Gaudiani, M. D., Clinical Associate, Clinic of Surgery, NHLBI
Philip O. Alderson, M.D., Major USAF, Naval Medical Center

COOPERATING UNITS (if any)
Armed Forces Radiology Research Institute

LAB/BRANCH
Surgery

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	3/4	1/4

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

This project documents the accuracy and reliability of quantitative radioangiocardiography (QRAC) in measuring left to right shunts in experimental atrial septal defects. This technique has been used clinically but has not been evaluated in an experimental model.

834

Description: Atrial septal defects were made in eleven healthy beagle dogs, and after recovery, ten QRAC determinations were made in each dog over a three week period. Six dogs had repeated cardiac catheterizations and shunt measurement by oximetry. Seven dogs had median sternotomy and shunt measurement by electromagnetic flow probe under three conditions: Control, PA banding, and LV obstruction.

Results show that QRAC gives more consistent results than oximetry in these animals. The correlation of flow probe and QRAC determinations was $r = 0.97$.

Proposed course: This paper has been accepted for publication in the Surgical Forum 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02629-02 SU

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Evaluation of Hemolysis Following Atrioventricular Replacement with Porcine Xenograft (Hancock) Valves

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

PI: Glen R. Rhodes, M.D., Surgeon, Clinic of Surgery, NHLBI

OTHER: Charles L. McIntosh, M.D., Senior Surgeon, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

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LAB/BRANCH
Surgery

SECTION
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INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1/2	1/2	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Hematologic and hemolysis studies were performed in 22 patients following xenograft atrioventricular valve replacements to evaluate the degree of hemolysis associated with these valves. Patients were studied 6-62 months postoperatively (mean 30.5 months). Preoperative mean hematologic values were: Hematocrit 39.4, reticulocyte index 1.3%, platelet count 281 x $10^3/mm^3$ and LDH 234. The LDH was greater than 400 in five patients. The postoperative mean values were: Hematocrit 40.8, reticulocyte index 1.2%, platelet count 227 x $10^3/mm^3$ and LDH 204. Hemolysis studies including serum hemoglobin, and serum haptoglobin failed to indicate hemolysis in any patient. Postoperative iron studies were normal for the majority of patients. Only two patients had mild anemias, and their hemolysis studies were normal. Five patients had significantly elevated LDH values preoperatively which returned to normal following operation. Our evidence suggests that the glutaraldehyde fixed porcine xenograft provides excellent hemodynamic function without significant thromboembolism or chemically detectable hemolysis. Presently, it is our valve of choice for all atrioventricular replacements.

836

Description: Intravascular hemolysis is a frequent and occasionally serious complication of prosthetic valvular replacements. A reduction in thromboembolism and hemolysis has been sought through clinical trials of new and modified prosthetic and tissue valves. Perhaps the most widely used tissue valve has been the glutaraldehyde-fixed porcine xenograft (Hancock) on a Dacron-covered flexible stent. In five years of experience, this valve has provided excellent hemodynamic function without significant thromboembolism. Also, it has been our clinical impression that negligible hemolysis occurs with this valve. To test this hypothesis, hematologic studies were performed on 22 patients following atrioventricular valve replacements.

Twenty-two patients who had undergone porcine xenograft replacement of one or more atrioventricular valves were investigated, either during their admission for postoperative catheterization or during their annual clinic visit. Blood was drawn for measurement of a complete blood count, reticulocyte index, red cell indices, and platelet count. LDH and total billirubin were determined by standard methods. Serum hemoglobin, serum haptoglobin, serum iron, total iron binding capacity, and iron saturation were also measured.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02632-02 SU

PERIOD COVERED July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Intraoperative, Intracardiac Echogram Measurements of Septal Thickness in IHSS: Correlation to Postoperative Results

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

PI: Donald C. Syracuse, M.D., Clinical Associate, Clinic of Surgery, NHLBI

OTHER: Vincent A. Gaudiani, M.D., " " " " "
David G. Kastl, M. D. " " " " "
Walter L. Henry, M.D., Senior Investigator, Cardiology Branch, NHLBI
Andrew G. Morrow, M.D., Chief, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

Cardiology Branch, NHLBI

LAB/BRANCH
Surgery

SECTION

INSTITUTE AND LOCATION
NHLBI-NIH-Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	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)

During left ventriculomyotomy and myectomy for IHSS, precision in making a trough has been enhanced by an intracardiac echo probe which allows exact measurements of septal thickness. Its routine use in patients with IHSS, but with a relatively thin septum, may lessen the risk of iatrogenic VSD. This study analyzes the depth of the myectomy as a contribution to the beneficial effects of the operation.

838

Description: Fifteen consecutive IHSS patients who had been catheterized preoperatively were studied with intraoperative echograms before and after myectomy. Correlations were made between intraoperative determinations of septal thickness and the postoperative results at 6 month catheterization.

Results: All 15 patients underwent 6 month recatheterization except 2 who died and one who refused further study. No early or late VSD's occurred. All patients were improved at least one functional class. The mean thickness of hypertrophic muscle excised was 9.9 mm $\pm$ .66 SEM. The mean thickness of the septum at the base of the trough was 14.3 mm $\pm$ .97 SEM. The mean percentage of septal thickness excised was 41.3% $\pm$ 2.84 SEM. No resting gradient occurred in postoperative patients. Most patients, however, still showed a provocable gradient, although lessened from the peak provocable gradient preoperatively (mean percentage decrease 70.3%).

The depth of the myectomy failed to correlate with the postoperative result. When the percentage septum excised was compared to the percentage change in provocable gradient, $r=-.32$, $p = .36$. When the remaining septal thickness was compared to the absolute value of the peak provocable gradient, $r = -0.54$, $p = .07$.

It seems likely that some other factors such as the length and width of the myectomy are more important determinants of the outcome of the operation than the depth of the trough. It appears that no advantage is served by extending the depth of the myectomy beyond approximately 10 mm. (mean septal thickness excised in this series) and a deeper myectomy only increases the risk of iatrogenic VSD.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02640-02 SU

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Continuous Echocardiographic Monitoring of Left Ventricular Function Following Cardiac Operation.

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

PI: Vincent A. Caudiani, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Walter L. Henry, M.D., Senior Investigator, Cardiology Branch, NHLBI
Andrew G. Morrow, M.D., Chief, Clinic of Surgery, NHLBI

OTHER: Donald C. Syracuse, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Richard J. Shemin, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Willard C. Whitehouse, Chief, TV Engineering Section, CC

COOPERATING UNITS (if any)

Cardiology Branch, NHLBI and TV Engineering Section, CC

LAB/BRANCH
Surgery

SECTION

INSTITUTE AND LOCATION

NHLBI - NIH - Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1½	½

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)

Last year we reported development of an echocardiographic transducer for suture placement on the left ventricle to monitor cardiac size and ejection fraction. Current work has shown that reproducible and accurate estimates can be made of diastolic volume, systolic volume, and ejection fraction over a range of inotropic states.

840

Description: In 10 foxhounds and 10 sheep the accuracy, reproducibility, and safety of this technique were assessed. Echocardiographic estimates of diastolic and systolic volume and ejection fraction correlated with angiographic measurements: $r = 0.87$, $r = 0.76$, and $r = 0.84$ respectively. Reproducibility studies showed that all ultrasound measurements could be made with less than 5% error. None of the animals studied showed significant myocardial injury.

Because of these encouraging results, clinical studies have been started. Six patients have been studied for up to 72 hours after operation. In three, the probe was removed percutaneously without incident. Data analysis is underway, but preliminary findings suggest that ultrasound measurements can provide a new insight into cardiac function immediately following cardiac operations.

Proposed course: Animal work has been accepted for publication in the Surgical Forum 1977. Preliminary human data will be submitted to the American Heart Association meeting.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02642-01 SU

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Sequential Echocardiographic Studies in Patients Undergoing Left Ventricular Myotomy and Myectomy

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

PI: Andrew G. Morrow, M.D., Chief, Clinic of Surgery, NHLBI

OTHER: Walter Henry, M.D., Cardiology Branch, NHLBI
Walter H. Merrill, M.D., Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)
Cardiology Branch

LAB/BRANCH
Surgery

SECTION
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INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2½	2	½

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)

Routine echocardiographic examination of all patients suspected of having idiopathic hypertrophic subaortic stenosis (IHSS) has proven to be a valuable diagnostic tool in this clinic. Determination of the septal to free wall ratio and the detection of systolic anterior motion of the anterior mitral valve leaflet are the most essential portions of the examination. The purpose of this study is to critically evaluate sequential echocardiograms taken of patients undergoing left ventricular myotomy and myectomy. These data will be correlated with pre- and postoperative cardiac catheterization data and the subsequent clinical course of each patient. It is hoped that this study will demonstrate the following: first, that preoperative echocardiographic measurements of the left ventricular septum and free wall correlate closely with the findings at operation and/or necropsy; secondly, that the close proximity of the anterior mitral leaflet and septum during systole can be quantitated and correlated with the degree of outflow tract obstruction as determined by cardiac catheterization; and thirdly, that echocardiography is a useful tool in following and assessing the long-term results of myotomy and myectomy.

842

Description: Sequential echocardiograms have been obtained in patients undergoing left ventricular myotomy and myectomy. The examinations have been performed preoperatively, in the first or second postoperative week, six months postoperatively, and sequentially during each followup clinic visit. All echocardiograms are of diagnostic quality, and they are being analyzed in detail by the same observer. These data will then be correlated with pre- and postoperative cardiac catheterization data from approximately the same time as the echocardiograms.

Results: Final results are pending completion of the echocardiographic analyses.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02643-01 SU

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Pericardial Effusion Following Open Heart Operations: Fact or Fiction

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

PI: David G. Kastl, M.D., Surgeon, Clinic of Surgery, NHLBI

OTHER: Walter Henry, M.D., Senior Investigator, Cardiology Branch, NHLBI
Walter H. Merrill, M.D., Surgeon, Clinic of Surgery, NHLBI
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Wayne M. Derkac, M.D., Surgeon, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)
Cardiology Branch, NHLBI

LAB/BRANCH
Surgery

SECTION
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INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2 1/2	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)

This study was undertaken to evaluate the presence and significance of pericardial effusions in postoperative open heart patients. One hundred consecutive patients received echocardiograms 7-10 days and 6 months following their procedure. Presence or absence of pericardial effusion was correlated with chest tube placement and number, and pericardial sac closure. Anticoagulation therapy and/or hematological disorders also were noted. This investigation currently lacks several patients for completion and results are pending data analysis.

844

Description: Pericardial effusions invariably occur immediately following open heart operations. Large bore drainage tubes are placed in multiple spaces including pleural cavities to prevent pericardial tamponade. Despite adequate tube placement and cessation of drainage, a significant number of patients develop postoperative low cardiac output syndrome attributed to recurrent and/or residual pericardial fluid which may or may not be discovered by subsequent echocardiograms.

To determine the presence and significance of pericardial effusion following open heart operation, one hundred consecutive patients received M-mode echocardiograms 7-10 days and 6 months following their procedure. Questionable effusions were better delineated with cross-sectional (two-dimensional) echocardiograms. Postoperative echocardiograms are not routinely performed. Therefore, analysis of the echocardiograms were not made by the attending staff unless the patient's clinical course deemed it necessary. Significant pericardial effusion was not assumed and each patient was discharged to resume a conventional postoperative course. Presence or absence of pericardial effusion was correlated with number and placement of chest tubes, and pericardial sac closure. Anticoagulation therapy, hematological disorders, and clinical course also were noted.

This investigation lacks several patients for completion and results are pending data analysis.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02644-01 SU

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Results of Combined Coronary Endarterectomy and Coronary Bypass for Diffuse Coronary Artery Disease

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

PI: Mark S. Hochberg, M.D., Surgeon, Clinic of Surgery, NHLBI
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Lawrence L. Michaelis, M.D., Senior Surgeon, Clinic of Surgery, NHLBI
Charles L. McIntosh, M.D., Senior Surgeon, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)
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LAB/BRANCH
Surgery

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INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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 treatment of diffuse distal coronary artery disease is presently unsatisfying. Coronary artery bypass grafting (CABG) is usually not successful in these circumstances. Mechanical endarterectomy of a distal coronary artery combined with CABG has been performed 26 times at the NIH. Followup catheterization six months after operation revealed that 14 of the 20 grafts studied were patent (70%). Sixteen endarterectomies were performed to the distal right coronary artery, two to the distal left anterior descending coronary artery, and one each to the circumflex and diagonal coronary arteries. The average flow in these patent grafts at the time of operation was 87 ml/min (range 30-200 ml/min). Intraoperatively, the four nonpatent grafts had average flows of 27 ml/min. All of these 20 patients had concomitant CABG's without endarterectomy to other coronary arteries. There were two perioperative myocardial infarctions. There were no early or late deaths.

PHS-6040
(Rev. 10-76)

Description: A literature survey shows that 605 CABG's constructed to endarterectomized coronary arteries have undergone postoperative catheterization. Four hundred and seventy (79%) of these grafts were patent from three months to two years following surgery. Histologic studies demonstrate that a neo-intima is formed over the endarterectomized surface. Neither thrombosis nor the recurrence of atherosclerosis seems to be the problem that many have feared.

Surgeons who perform coronary bypass grafts are often unfortunately surprised to find an angiographically attractive artery unsuitable for bypass upon exploration in the operating room. Endarterectomy of coronary arteries alone has not proved to be of lasting value for this problem. However, the present series, as well as the combined series from the literature, lends encouraging support to the value of endarterectomy plus coronary artery bypass grafting for the treatment of a diffusely diseased distal coronary artery.

Presented at the American Association for Thoracic Surgery - 57th Annual Meeting, Toronto, Ontario, Canada, 1977. Accepted for publication in The Journal of Thoracic and Cardiovascular Surgery.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02645-01 SU

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Cross-sectional Echocardiographic Studies of the Mitral Valve Before and After Mitral Commissurotomy

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

PI: David G. Kastl, M.D., Surgeon, Clinic of Surgery, NHLBI

OTHER: Walter L. Henry, Senior Investigator, Cardiology Branch, NHLBI
Charles L. McIntosh, M.D., Senior Surgeon, Clinic of Surgery, NHLBI
Andrew G. Morrow, M.D., Chief, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)
Cardiology Branch, NHLBI

LAB/BRANCH
Surgery

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INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1 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)

Increasing emphasis has been placed on diagnosing and evaluating cardiac dysfunction with noninvasive methods. Mitral stenosis has been evaluated for years with standard M-mode echocardiograms, however, the degree of specificity of orifice narrowing has been notoriously poor in tight stenosis. Recent technical advances have resulted in the development of cross-sectional (two dimensional) echocardiographic images which allows direct measurement of the mitral orifice area. This study correlated the latter method before and after mitral commissurotomy with concomitant hemodynamic evaluation of the mitral valve area in patients with mitral stenosis.

8K8

Description: Congentional M-mode echocardiography is a simple, non-invasive technique for diagnosing mitral stenosis. However, it has not proved reliable in quantifying the severity of mitral stenosis nor in assessing the degree of residual stenosis after mitral commissurotomy. Cross-sectional echocardiography provides a direct image of the mitral orifice that enables quantitative assessment of the mitral valve area. Its usefulness in assessing the results of mitral commissurotomy was therefore evaluated.

Ten patients with significant mitral stenosis underwent pre- and postoperative M-mode and cross-sectional echocardiogram with concomitant complete cardiac catheterization. Successful mitral commissurotomy was performed in all patients as demonstrated by hemodynamic parameters and return to a NYHA functional Class I. Comparison between the standard M-mode parameters with either the Gorlin formula or cross-sectional valve area confirmed the poor correlation reported by other investigators.

The cross-sectional measurement of mitral orifice area correlated well with the area measured by the Gorlin method ($r = 0.91$). The two independent measurements of mitral orifice area were related by the linear regression equation: CROSS SECTIONAL AREA = 0.98 (Gorlin Area) + 0.3.

It was therefore concluded that noninvasive cross-sectional echocardiographic assessment of mitral orifice size is a valuable method for evaluating patients preoperatively as well as for assessing the results of mitral commissurotomy.

Results: Manuscript submitted for publication.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02646-01 SU

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Aortic Valve Replacement in the Elderly: Encouraging Postoperative Clinical and Hemodynamic Results

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

PI: Mark S. Hochberg, M.D., Clinical Associate, Clinic of Surgery, NHLBI
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Charles L. McIntosh, M.D., Senior Surgeon, Clinic of Surgery, NHLBI

Others: Stephen Epstein, Chief, Cardiology Branch, NHLBI
Kenneth Kent, Senior Investigator, Cardiology Branch, NHLBI

COOPERATING UNITS (if any)

Cardiology Branch, NHLBI

LAB/BRANCH
Surgery

SECTION

INSTITUTE AND LOCATION
NHLBI-NIH-Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
21	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)

From 1966 through 1975, 73 patients aged 60 years and over and 277 patients under 60 underwent isolated aortic valve replacement (AVR) for aortic stenosis (AS), regurgitation (AR) and mixed disease (AS/AR). Cardiac catheterization was performed 5-9 months following operation in 77% of these patients. Followup averaged 55 months per patient.

850

PHS-6040
(Rev. 10-76)

Description: The hospital mortality in the elderly group was 2.7%, compared with 5.8% in the younger group. The late cardiac mortality was 21% and 18%, respectively. There was significant improvement ($p < .001$) in the LVEDP, cardiac index, and functional class in each of the three disease groups (AS, AR, AS/AR) and peak systolic gradient in the AS group in the younger as well as in the elderly patients. More importantly, the magnitude of improvement in these parameters in patients over and under 60 years did not differ at the standard ($p = < .05$) significance level.

Increasing longevity will make cardiac surgery more common in the older population. These things indicate that AVR carries the same low risk and brings about a similar improvement in left ventricular pump function in patients older and younger than 60 years. This paper was presented at the annual meeting of the International Cardiovascular Society in June 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02647-01 SU

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Hemodynamic Response to Rhythm in IHSS

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

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Donald C. Syracuse, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Richard J. Shemin, M. D., " " " " "
William C. Scott, M.D., " " " " "

COOPERATING UNITS (if any)
Cardiology Branch

LAB/BRANCH
Surgery

SECTION

INSTITUTE AND LOCATION
NHLBI-NIH-Bethesda, Md. 20014

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)

A noncompliant left ventricle is a common finding in patients with idiopathic hypertrophic subaortic stenosis (IHSS). Secondarily, arrhythmias produce symptomatic reduction of the cardiac output, which is seldom tolerated. Following left ventricular myotomy and myectomy with complete reduction of the LV-aortic gradient and significant improvement in the quality of life, these patients continue to tolerate poorly arrhythmic disturbances.

852

Description: Ten candidates for operative palliation of IHSS are currently undergoing atrial and ventricular pacing studies pre- and post-operatively to relate these rhythms to cardiac performance. Concomitant left ventricular hemodynamic evaluations are performed with determination of cardiac output, systemic blood pressure, and pulmonary wedge pressure.

Results are pending completion of the study population and data analysis.

Idiopathic hypertrophic subaortic stenosis (IHSS) is a disease characterized by marked asymetric hypertrophy of the left ventricle, involving in particular the interventricular septum and left ventricular outflow tract. Considerable emphasis has been given to the clinical and hemodynamic understanding of IHSS. However, little information regarding cardiac rhythm and its effects on the circulation has been available, especially regarding reduction of the LV-aortic gradient.

To better understand the relationship of rhythm and circulation response, and the postoperative management of left ventricular myotomy and myectomy patients, ten candidates for operative palliation are undergoing atrial and ventricular rhythm evaluation. Preoperative and postoperative pacing studies are performed monitoring several cardiovascular hemodynamic parameters. Each rhythm is evaluated independently of the other at different rates above the intrinsic rate. Concomitant cardiac output, systemic blood pressure, and pulmonary wedge pressure determinations are recorded. The preoperative study is conducted during cardiac catheterization and postoperative evaluation is performed 24-48 hours following the procedure utilizing a Swan-Ganz catheter and routinely operative placed pacing wires. Following cardiopulmonary bypass pumping the LV-aortic gradient is measured and recorded intraoperatively. This investigation is currently being conducted and results are pending data analysis of the complete study population.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 02648-01 SU

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Atrial Patch Enlargment of the Right Ventricular Outflow Tract

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

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William C. Scott, Surgeon, Clinic of Surgery, NHLBI
Vincent A. Gaudiani, Surgeon, Clinic of Surgery, NHLBI
Thomas L. Spray, Clinical Associate, Pathology Branch, NHLBI
David, M. Conkle, Senior Surgeon, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

Pathology Branch, NHLBI

LAB/BRANCH

Surgery

SECTION

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INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	.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)

An autograft fashioned from the right atrial appendage was evaluated as a right ventricular outflow patch. Theoretically, such an autograft would resist aneurysm formation, as seen with pericardial patches, and lessen the risk of endocarditis, as seen with prosthetic materials. The autograft was harvested at the site of caval cannulation. It assumed a diamond shape, measuring 35 mm x 30 mm, with the long axis formed by the crista terminalis.

854

Description: Twelve foxhounds underwent right ventricular outflow patching with atrial autografts. All dogs survived the operation without evidence of patch leakage or rupture. All dogs remained in sinus rhythm. Eight dogs were studied with catheterization, angiography, and histology at intervals of one to eight weeks following operation. Four dogs are alive nine months postoperatively; they were studied every two months following operation.

Results: At all intervals following the operation, catheterization showed normal RV pressures (mean 12.8 $\pm$ 2.1 mm Hg) and angiography showed no stricture or aneurysm of the outflow patch. Histology showed sequential fibrous tissue replacement of atrial muscle cells. The trabeculations of the atrial appendage were molded to smooth contours within one month. No thrombus was evident. Scar formation did not distort outflow tract anatomy.

The atrial patch graft is offered as an adequate option for widening the RV outflow tract. It handles easily, is routinely available on caval cannulation, and may provide some advantages over currently used patch materials.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02649-01 SU

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Glutaraldehyde Preserved Vascular Grafts

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

PI: David G. Kastl, Surgeon, Clinic of Surgery, NHLBI

OTHER: Thomas Spray, Clinical Associate, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

Pathology Branch, NHLBI

LAB/BRANCH
Surgery

SECTION
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INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1 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)

This study was undertaken to evaluate glutaraldehyde preserved vascular xenografts as possible vascular substitutes in humans. Projected utilization was aimed towards replacing or bypassing arterial lumen diameters 4 mm or less and as possible arteriovenous shunts for renal dialysis. Two groups of adult dogs were acquired for short-term study. Group A received right femoral glutaraldehyde preserved interposition xenografts. In the same animal the left femoral artery was similarly replaced with an autogenous venous graft. Group B animals received femoral arteriovenous shunts constructed with glutaraldehyde preserved xenografts. All grafts remained intact for 3-8 weeks at which time they were reclaimed for histological examination. No femoral artery imposition grafts were patent, however, all contralateral venous grafts remained patent for the complete duration. All arteriovenous shunts were patent for one week after which most occluded at varying intervals.

852

PHS-6040
(Rev. 10-76)

Description: Saphenous vein grafts have been utilized as an arterial substitute to replace damaged vessels, bypass atherosclerosis occlusions, and to construct arteriovenous shunts for renal dialysis. However, many patients have insufficient veins due to deficiencies, disease, and/or previous harvesting. In addition, many vein grafts produce subendothelial cellular proliferation in the arterial position leading to graft failure in some cases. Also, the venous arteriovenous shunts tend to form false aneurysms and/or failure with repetitive puncture. Dacron woven grafts have shown only excellent applicability with large lumens and certainly not as renal dialysis shunts.

In cooperation with Hancock Laboratories, Inc. (Anaheim, California) the present study utilized two groups of adult dogs to evaluate glutaraldehyde preserved bovine vascular xenografts. Group A consisted of 12 femoral interposition xenografts with end-to-end anastomoses. The contralateral femoral artery of each animal received an autogenous venous interposition graft constructed in the same fashion to serve as a control. Twenty femoral arteriovenous shunts were constructed with the glutaraldehyde preserved vascular conduits with end-to-side anastomoses in Group B.

All grafts were reclaimed at varying intervals between 3-8 weeks. No femoral interposition graft was patent at one week but all contralateral venous grafts were patent at 8 weeks. All arteriovenous shunts were patent at one week but the majority occluded at varying intervals within 8 weeks. Only six arteriovenous shunts remained patent for the duration of the study.

Gross examination at eight weeks revealed intact xenografts with little adjacent tissue reaction and no infection by culture. No anastomosis had a false aneurysm nor evidence of leakage. Histological examination of all xenografts showed minute foreign body reaction and occasional giant cell clusters. Each anastomosis was adequately constructed with excellent eversion of the edges and no obvious nidus for thrombosis. However, each occluded graft revealed an organized thrombus attached only to the proximal anastomosis. There was no endothelization of any xenograft lumen. In addition, varying degrees of focal calcification were noted in most xenografts which appeared to originate from the medial portion of the graft wall.

Although the early patency rate of glutaraldehyde preserved xenografts was not significant, as a pilot study the results were encouraging. Several similar vascular prototypes by other investigators have results in total early failure secondary to high thrombogenicity and foreign body reaction. The current product needs further investigation of tissue preservation but has shown good durability and inertness to host tissue.

Result: The present study has served as a guide for further research in tissue preservation and graft thrombogenicity. Currently additional vascular xenografts are being prepared for transplantation with proposed improvements.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02650-01 SU

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Limits of Myocardial Protection with Potassium Cardioplegia

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

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William C. Scott, Surgeon, Clinic of Surgery, NHLBI

OTHER: Vincent A. Gaudiani, Surgeon, Clinic of Surgery, NHLBI
David M. Conkle, Senior Surgeon, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Surgery

SECTION
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INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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)

This project stuides the duration and degree of left ventricular preservation that can be obtained with potassium cardioplegia during aortic crossclamp (Ax). Potassium was the sole cardiolegic substance in the normal saline solution.

858

Description: Left ventricular function was evaluated by constructing ventricular function cruves and by measurement of VPM (dp/dt/P max). Twenty-six foxhounds divided into five groups underwent ventricular function evaluation before and after vented cardiopulmonary bypass at 30°C. Groups I and II served as controls. In Groups III, IV, and V a single bolus of cardioplegia solution (150 cc, isotonic, 25 mEq K+/L in NS) was injected into the aortic root after aortic crossclamp.

Results:

Group	Intervention	*VFC Avg. Depression	%Change of VPM ± SE
I	45 minutes - No Ax	1.4 ± 0.6	+1.0 ± 0.5
II	45 minutes - Ax	5.0 ± 0	-60.5 ± 0.5
III	45 minutes - Ax - K+	2.0 ± 1.2	-7.2 ± 4.3
IV	60 minutes - Ax - K+	3.3 ± 1.9	-25.8 ± 11.4
V	75 minutes - Ax - K+	5.0 ± 0	-52.5 ± 2.6

*Degree of depression of VFC was graded 1 = No change, 2 = Minimal, 3 = Moderate, 4 = Severe, 5 = Unable to wean from CPB.

The data demonstrates that the preservation of LVF during K+ cardioplegia is excellent at 45 minutes, inconsistent at 60 minutes, and poor at 75 minutes. Thus, the degree and time limit of preservation one may consistently expect from a cardioplegic solution containing K+ alone is limited to 45 minutes.

857

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02651-01 SU

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Alterations in Regional Contractility Following Cardiopulmonary Bypass with Intraoperative Ischemia

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

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Stephen E. Epstein, Chief, Cardiology Branch, NHLBI

COOPERATING UNITS (if any)

Cardiology Branch, NHLBI

LAB/BRANCH

Surgery

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INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	2/3	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)

This project documents the effects of local coronary occlusion during cardiopulmonary bypass on postbypass regional ventricular function under a variety of conditions. The experimental model used parallels a clinical situation encountered during saphenous vein coronary artery bypass grafting.

860

Description: Twenty dogs were placed on cardiopulmonary bypass and regional function was examined using ultrasonic crystals during and after ten and thirty minute periods of left anterior descending coronary artery occlusion. The experiments were designed to compare fibrillating versus beating hearts and normothermic versus hypothermic (30°C) hearts.

Results show that after ten minutes of ischemia during normothermic total cardiopulmonary bypass, only fibrillating hearts show a significant depression of regional contractility. After thirty minutes of ischemia with the same conditions both beating and fibrillating hearts are depressed. When hypothermic bypass is used, thirty minutes of ischemia produces depression only in fibrillating hearts.

Proposed Course: This work is complete and will be submitted to the Journal of Thoracic and Cardiovascular Surgery.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02652-01 SU

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Coronary Venous Bypass Grafts: Long-term Hemodynamic Evaluation with Radioactive Microspheres

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

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David M. Conkle, Senior Surgeon, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
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INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.75	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)

Selective retrograde coronary venous perfusion (RCVP) was performed to the left anterior descending vein (LADV) in 14 Foxhound dogs. A saphenous vein was interposed between the aorta and LADV. The LADV was ligated proximal to the coronary venous bypass graft (CVBG) to prevent an A-V fistula. The left anterior descending artery (LADA) was also ligated at its origin to precipitate an acute infarction.

862

Description: The dogs were catheterized 3-5 months following operation. Ten of the 14 CVBG's were angiographically patent. The chests were then opened and graft flow measured 50.7 ml/min compared to measured operative flows of 53.0 ml/min. ^{141}Ce microspheres were next injected into the left atrium to measure RCVP perfusion to the subendo-, myo - and sub-epicardium. The CVBG was then ligated and ^{85}Sr was injected. RCVP full thickness anterior wall average flow was 39.3 $\pm$ 2.8 ml/100 gm -tiss/min in the 10 patent grafts. The average subendocardial flow in these 10 hearts was 39.4 $\pm$ 11.3. Next the grafts were ligated, and flow dropped to 15.2 $\pm$ 4.2. In 15 control dogs, the normal anterior wall flow was 100 $\pm$ 9.7 ml/100 gm tiss/min. Acute ligation of the LADA in these controls dropped flow to 13.5 $\pm$ 5.8.

RCVP can improve anterior wall flow almost threefold when compared to the ischemic state of LADA ligation. Moreover, this restoration of flow to an ischemic LV is effective because it perfuses the subendocardium - the most crucial layer of myocardial muscle.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02653-01 SU

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Development of a Microcrystal Echoprobe for Chronic Implantation on Vessel Walls

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

PI: Donald C. Syracuse, Surgeon, Clinic of Surgery, NHLBI

OTHER: Vincent A. Gaudiani, Surgeon, Clinic of Surgery, NHLBI
David G. Kastl, Surgeon, Clinic of Surgery, NHLBI
Mark S. Hochberg, Surgeon, Clinic of Surgery, NHLBI
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COOPERATING UNITS (if any)

Cardiology Branch

LAB/BRANCH

Surgery

SECTION

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INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1/4	1/4	

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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)

A 1.5 mm, tear-drop shaped piezoelectric crystal (2.5-3 mHz) is small enough to place at a side branch of a grafted vessel and send signals across the lumen of the trunk vessel. Theoretically, the echo imaging of a vessel with flow would be different from a vessel without flow and would be useful in determining the patency of grafted vessels in the chest as well as in the periphery.

864

Description: Echo crystals with various design features are being placed on both veins and arteries of foxhounds. Fine wires (000) lead from the crystals and are buried beneath the skin. A-mode echograms are recorded daily from the crystals.

Results: The crystals give near-field images which correlate with the size of the vessel lumen. Arterial pulsations are clearly visible on echograms. When a vessel is obstructed and made to dilate, the echogram correspondingly shows luminal wall separation. The image has been reproducible through five days, at which time the crystals are retrieved and examined for structural change. Difficulties have been encountered in maintaining the crystal in a fixed position on the vessel, but the problem is less now that a side branch is used for crystal tethering. It appears that within a 5-day period, no damage occurs to the vessel wall and the crystal remains sealed from body fluids.

The next phase of these experiments will involve thrombosis of the vessel. It is expected that use of the crystal will allow early diagnosis of occluded grafts or reconstructed vessels, thereby lessening the need for arteriography and permitting earlier deployment of definitive therapy.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02654-01 SU

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Glutaraldehyde Preserved Tracheal Xenografts

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

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OTHER: Thomas Spray, Clinical Associate, Pathology Branch, NHLBI

COOPERATING UNITS (if any)
Pathology Branch, NHLBI

LAB/BRANCH
Surgery

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INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1 1/4	1	1/4

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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)

Glutaraldehyde preserved porcine tracheas were implanted in 12 adult dogs to be evaluated as tracheal and bronchial prostheses. No respiratory problems were encountered during the first postoperative week. Afterwards, rapid stricture formation was noted at each anastomosis with greater progression at the proximal anastomosis. The anterior portion of each anastomosis also showed more stricture involvement by bronchoscopy and cervical soft tissue x-ray. At autopsy four weeks later, no gross contamination was encountered and the xenograft was densely encased in fibrotic tissue. No anastomotic leakage or graft disruption was noted. Histological examination showed marked foreign body reaction and focal "necrosis" of the xenograft. Significant stenosis (60% or greater) was noted at each anastomosis (proximal > distal) secondary to marked tracheal inflammation and host-graft reaction.

866

Description: Prolonged use of endotracheal tubes and construction of tracheostomies has been associated with significant morbidity due to tracheal stricture. Dilatation, multiple tissue debridement, and tracheal excision with a primary anastomosis have improved respiratory related problems. However, a significant number of patients have greater stricture formation or destruction not amenable to these methods. In addition, many pulmonary lobectomies are needlessly performed due to cancer involvement of major bronchial branches.

Advancement in tracheal and bronchial reconstruction has not produced an adequate prosthesis. This study was undertaken in cooperation with Hancock Laboratories, Inc. (Anaheim, California) to evaluate glutaraldehyde preserved porcine tracheal implants in adult dogs. Twelve animals underwent removal of a four centimeter mid-tracheal segment which was replaced with a tracheal xenograft. Although tracheal procedures are not considered strictly sterile procedures, no wound infections were apparent. After one week the first three animals asphixiated secondary to sloughing of the xenograft epithelium. Subsequently the lining was removed prior to implantation. In the remaining nine animals, rapid and progressive stricutre formation at the anastomoses was followed with bronchoscopy and cervical x-rays. The proximal anastomosis and the anterior portion of both anastomoses had the most involvement. Marked stridor and respiratory distress was apparent at four weeks and all animals were killed.

At autopsy the xenografts were densely encased in fibrotic tissue and no disruption or anastomotic leakage was noted. The tracheal lumens were stenosed 60% or greater at the anastomoses - specifically the proximal anastomosis in each animal. Histology examination revealed focal necrosis of the xenograft and marked foreign body reaction. The stricture formation consisted of inflammatory and giant cells. In addition, early cartilaginous calcification was noted histologically and by radiological examination in vivo.

Results: Inadequate tissue preservation has prevented further research in this area. Currently Hancock Laboratories has undertaken steps to improve the tracheal preservation techniques, removing the epithelial lining, and constricting a tracheal carcass completely circumscribed by cartilagenous rings for continued investigation.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02655-01 SU

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Hemodynamic Evaluation of Right Atrial to Pulmonary Artery Bypass Conduits for Tricuspid Atresia

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

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OTHER: David M. Conkle, Senior Surgeon, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Surgery

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INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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)

Recent clinical success with right atrial to pulmonary artery bypass conduits for tricuspid atresia stimulates interesting questions regarding the physiology of blood flow through the conduit. Since the right atrium becomes the sole pumping chamber on the right side of the heart, it would seem that normal sinus rhythm would be required for optimal flow through the conduit. However, there have been reports of patients adequately tolerating nodal rhythm as well as supraventricular arrhythmias. Thus, the importance of sinus rhythm for hemodynamic success is not clearly established. Furthermore, the necessity of including a valve in the conduit has been questioned.

PHS-6040
(Rev. 10-76)

Description: The initial aim of this project is to create a colony of foxhounds with tricuspid stenosis using a silastic patch material sutured in the tricuspid valve orifice. This should allow right atrial dilatation and hypertrophy as well as provide time for the dogs to adjust hemodynamically to elevated central venous pressures. Right heart catheterizations have subsequently demonstrated right atrial enlargement and elevated right atrial mean pressures. The second part of the project consists of the performance of a right atrial to pulmonary artery anastomosis using a double-limbed conduit (one limb containing a valve, the other limb nonvalved). A purse string suture placed at the initial operation and left in the pericardium will be tied, thereby closing the tricuspid orifice completely. Then the hemodynamics of blood flow via the valved and nonvalved limbs will be studied under varying conditions of filling pressure, rhythm disturbances, and the phases of respiration.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02656-01 SU

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Myocardial Protection With Continuous Infusion Potassium Cardioplegia

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
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INSTITUTE AND LOCATION
NHLBI, NIB, Bethesda, Maryland 20014

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)

Myocardial protection during aortic occlusion (AO) utilizing potassium chloride cardioplegia was evaluated in 24 dog experiments. Ventricular function (Sarnoff curves) was compared before and after one hour of vented cardiopulmonary bypass (CPB) at 30°C. in five groups.

870

PHS-6040
(Rev. 10-76)

Description: Nine dogs (Groups I & II) were controls described below. Fifteen dogs were immendiately arrested at the time of AO with KCL (20 mEq/L) in saline at 30°C administered continuously through a catheter in the aortic root at 25 cc/min. This method eliminated collateral washout caused by noncoronary myocardial flow and maintained the heart at constant temperature. Group III received KCl-saline alone. In Group IV Krebs buffer (pH = 7.4) was added, and in Group V buffer and glucose (250 mg%) were added. The osmolarity of these solutions was comparable.

Results: The degree of ventricular depression and the number of animals is summarized below.

Groups	Number of Animals and Degree of Ventricular Depression		
	Minimal	Moderate	Severe
I - AO	-	-	5
II - No AO	4	-	-
III - AO - KCl - saline	1	2	2
IV - AO - KCl - buffer - saline	1	3	-
V - AO - KCl - buffer - glucose - saline	3	3	-

In representative animals from Groups III, IV, and V radioactive microspheres were introduced to estimate perfusate distribution to the myocardium. The ratio of endocardial to epicardial flow was 1.2 $\pm$ .2 in III, 1.2 $\pm$ .3 in IV and 1.6 $\pm$ .5 in V.

In conclusion, KCl in any solution is significantly more effective in preventing severe ventricular depression than AO alone at 30°C ($p < .05$). Buffered KCl completely prevents severe depression of function, but the benefit of providing glucose was not shown. Continuous low flow perfusion of the coronary bed is favorably distributed to the myocardim. The major factors protecting the heart during aortic occlusion in this model are immediate arrest and the prevention of myocardial acidosis.

ANNUAL REPORT OF THE
LABORATORY OF EXPERIMENTAL ATHEROSCLEROSIS
NATIONAL HEART, LUNG AND BLOOD INSTITUTE
July 1, 1976 through September 30, 1977

The lethal lesion of atherosclerosis is characterized by a usually well-circumscribed region of intimal thickening which is characterized histologically by the presence of smooth muscle cell hyperplasia, connective tissue proliferation, and the interstitial deposition of various lipid substances, predominantly cholesterol and cholesterol ester. Although study of the role of smooth muscle cell proliferation in atherogenesis is currently in vogue, there is no evidence that proliferation in the absence of lipid deposition contributes to morbidity and, in fact, under experimental conditions appears to be mostly a reversible process. Although repeated bouts of endothelial injury followed by intimal smooth muscle hyperplasia and regression are probably reflected in the common occurrence of intimal fibrosis seen with aging, it is the sequence of events leading to intimal lipid deposition that appear to be cardinal factors in the development of the lethal lesion of atherosclerosis.

Therefore, the activities of this laboratory have emphasized study of the processes that could lead to abnormal deposition of lipid in the intimal space. Viewed in its broadest terms, intimal lipid deposition may be considered in terms of an unbalanced set of transport processes and associated local metabolic processes. More specifically, it appears that atherogenic precursors are being transported into the intimal space and metabolized more rapidly than the cholesterol-rich metabolites can be transported out of the space. This simple scheme provides the working hypothesis around which the program of this laboratory has been designed. For purposes of this report, these studies may be summarized under the headings of transport, atherogenic precursors, and arterial metabolism.

Transport: Lipoidal atherogenic precursors as well as the lipid soluble metabolites of these must be transported through the various aqueous phases of the body in some water soluble or miscible form. This requires the formation of various complexes and vehicles which can interface with the lipid on the one hand and aqueous milieu on the other. Phospholipids, bile acids, soaps, fatty acids, and many proteins are examples of such substances. The soaps, bile acids, and fatty acids are transported on albumin along with certain other lipid moieties. The apoproteins of the lipoproteins appear to serve this function for the bulk transport of lipid. In any event, most of the different vehicles for lipid transport are macromolecules. Since albumin may play a central role in lipid transport, directly or indirectly, and since albumin is a well-defined stable molecule for study, it has been chosen as a model macromolecule upon which we have focused in our experiments so that we can discover and define the nature and controlling parameters of the various components of macromolecular transport through aqueous and arterial tissue phases. Six of the progress reports this year bear directly on these activities and the reader is referred to these for more detail. These are summarized briefly as follows.

Our transport studies can be divided into two sets of inquiry, one to establish the relevance of increased vascular permeability to atherogenesis and the other to characterize this altered permeability in terms of physically and mathematically definable transport processes. The relevance of altered

permeability to the subsequent development of atherosclerosis was determined by mapping the topographic pattern of altered permeability along the arterial tree in normal animals and noting the congruence of this pattern of increased permeability with the pattern of intimal lipid deposition that occurred in matched groups of animals on an atherogenic regimen. These studies were carried out in colonies of dogs, swine, and primates. It was found that a striking congruence between increased permeability in the normal group of animals and the pattern of subsequent sudanophilia in the experimental animals occurred. However, the most severe atheromatous disease did not always occur in the areas of greatest permeability indicating that an increased influx of atherogenic precursors only increases the propensity for the disease and that other local factors in transport or metabolism, as yet undefined, are also playing a significant role.

These studies required other preliminary work to develop methodology for measuring the topographic pattern of altered permeability in living animals. The use of radiolabeled proteins, in the present case albumin, is completely impractical from the point of view of measurement and is objectionable from the point of view of environmental considerations, particularly when dealing with large numbers of large animals. Therefore, an alternate method using the isomer of Trypan Blue dye, Evans blue (T1824), which binds tightly to albumin was devised. Studies were carried out comparing simultaneous uptake of EBD complexed albumin (EBD-alb) and radiolabeled albumin (I-alb) by the normal and the deendothelialized arterial wall. It was found that a monotonic relationship existed between the uptake of the EBD-alb and the I-alb; however, this was different for the normal surface as compared to the injured surface. The relationship was linear with time for the normal surface and markedly curvilinear for the injured surface for both labeled quantities. Moreover, the uptake of EBD-alb was four times greater in the injured and only half of that for I-alb in the normal surface. These results showed that while EBD is almost entirely complexed with albumin in the plasma phase, upon entry into the arterial phase there is a competitive binding for the dye between albumin and certain fixed structural substances of the wall. In the presence of a normal endothelial surface, the uncomplexed albumin is preferentially transported into the wall indicating that the reflection coefficient for EBD is greater than that for albumin. While these observations are of academic interest and importance, and also are of interest as possible prototype systems for study of lipid transport and binding in the arterial tissue phase, they are of most practical significance in showing that a monotonic and definable relationship does exist between the transport of EBD into the wall and the transport of albumin. This validates the use of EBD, a much more easily measured and environmentally acceptable substance, as a measure of the accessibility of the intimal space to various plasma macromolecules.

For parenterally administered EBD to be a useful approach to mapping the topographic distribution of altered endothelial permeability, methodology had to be developed for its measurement. A reflectance technique was developed (published this year) describing in detail the methodology as well as the validation of this measurement. The development of this reflectance technique as well as the establishment of a monotonic relationship between EBD accumulation and albumin accumulation represents a major advance in our ability now to quantify the role of altered endothelial permeability in atherogenesis as well as other arterial disease processes.

We made major advances in our understanding of detailed transvascular transport in arterial tissue this year as well as advances in new methodology

for the discrete measurement of distribution of radiolabeled proteins across the arterial wall. The transport of albumin across the arterial wall appears to be governed by two processes, diffusive and convective. It was found that, in the absence of a pressure across the wall, the principal driving force for transport was diffusive and that, in the absence of an endothelial surface, the uptake of radiolabeled albumin followed an essentially "square root of time law" indicating that the wall was behaving (to a first approximation) as a "homogeneous slab" model for diffusion. In the presence of normal endothelial surface, the uptake was found to be linear with time. Moreover, in a period of 1 hour about five times less albumin enters the normal wall than enters the deendothelialized wall. This indicates that the endothelial surface acts as a discrete but very large barrier to the transport of protein from the plasma phase to the arterial phase.

When a transmural pressure of approximately 100 mm Hg is imposed across the wall, a significant convective force to transport is added to the diffusive force thereby increasing the uptake of labeled albumin. A special technique was devised whereby the influences of these separate driving forces could be separated and measured. It was found that in the deendothelialized wall the convective transport was of the same order of magnitude as the steady state diffusive transport. Thus, for the first time, it was shown that an increased arterial pressure increases the influx of plasma substance into the arterial wall, as has been assumed from clinical observations but never measured. Similar studies are currently being done for the intact endothelialized artery. The significance of these studies is that it allows quantification of the role of both altered pressure states as well as altered diffusive states so that we now have another tool for gaining deeper insight into the mechanisms of atherogenesis and its relationship to hypertension.

Considerably more information regarding transport processes through arterial tissue can be gained by studying the discrete concentration of a particular radiolabeled protein across the arterial tissue under experimentally imposed constraints of time, concentration, and manipulated physiologic or pharmacologic states. Until now, technology for this has been possible only under very special experimental circumstances. We have developed methodology so that these important measurements can be done now in a variety of in vitro and in vivo experimental configurations and with a very high degree of resolution (of the order of 10 μm). To do this, quantitative autoradiographic techniques were developed in which the local concentration of silver in the developed autoradiograph is measured by scanning across the micrograph with an electron microprobe. These experiments demonstrated that the relationship between the concentration of radiolabeled protein at any point in the wall is directly proportional to the signal from the electron microprobe. The initial application of this new methodology has been to determine the distribution of radiolabeled albumin across deendothelialized aortic and iliac arterial walls in the quasi steady state and in the absence of a transmural pressure. The results of these studies have shown that the partition coefficient for radiolabeled albumin is essentially constant across the arterial wall, that the principal of superposition applies to diffusive transport of albumin through aortic tissue, and that the coefficient of diffusion for albumin in arterial tissue varies at certain sites across the wall in relation to various histologic structures. The significance of these studies, apart from the development of an experimental tool that should be applicable to other tissue systems for measurement of radiolabeled protein distributions, is that we can begin to study the parameters of macromolecular transport across the arterial

wall in sufficient detail that for the first time it should become possible to model these processes by physical and mathematical laws. This should permit inferences regarding detailed distributions of various macromolecules, such as atherogenic precursors, across the arterial wall from relatively simple measurements of uptake. Secondly, since this methodology provides access to detailed events within the wall, it opens the way to study of the equally important transport processes of cholesterol-rich metabolites out of the wall.

Atherogenic precursors and arterial wall metabolism: Comparative studies of dietary induced atherosclerosis in dogs, miniature swine, and Patas monkeys have allowed us to define the metabolic parameters necessary to produce arterial disease with characteristics resembling human atherosclerosis and have also provided new insights into some of the processes involved in the genesis of this important disease. The source of the dietary fat, fed in association with cholesterol, has been linked to the type, distribution, severity, and complications of the atherosclerosis. Cholesterol-rich diets which contain saturated fats, such as beef tallow, cause severe atherosclerosis that is frequently associated with thrombosis, particularly in dogs. Diets which contain unsaturated fats, e.g., cottonseed oil, result in a much less fulminant disease and no thrombosis. Our studies provide for the first time a reproducible model in which to study the role of the hemostatic processes, including platelet physiology, in the initiation and/or progression of the disease process. Changes in platelet membrane fluidity resulting from altered fatty acid composition and cholesterol content is thought to be responsible for sensitization of platelets to aggregation and thrombus formation. Studies are in progress to define the lipid composition of the membranes of platelets from animals on the various dietary protocols and to attempt to correlate these findings with changes in platelet function. Preliminary studies suggest that platelets from dogs on a saturated fat-cholesterol diet are promptly "sensitized" to aggregation whereas platelets from dogs on an unsaturated fat-cholesterol diet are not.

Detailed characterization of the plasma lipoproteins associated with cholesterol feeding in dogs, miniature swine, rats, rabbits, and Patas monkeys has led to the identification of certain consistent features of the atherogenic hyperlipoproteinemia. These include: 1) the production of a lipoprotein similar to the B-VLDL which occurs in human Type III hyperlipoproteinemia; 2) an increase in the low density lipoproteins and the intermediate lipoproteins which may arise from VLDL catabolism; and 3) the identification of a previously undescribed lipoprotein referred to by us as HDL_C. Associated with all of these lipoproteins is an increased prominence of a specific apoprotein protein, the "arginine-rich" apoprotein. Characterization of these lipoproteins and their apoproteins in the various species mentioned above is now completed. Studies are in progress to determine the origin and metabolic fate of these lipoproteins. Particular emphasis is being placed in the role of the "arginine-rich" apoprotein and the HDL_C in the regulation of cholesterol metabolism.

One of the important characteristics of the HDL_C, that is also shared with LDL, is the ability to be bound and degraded by skin fibroblasts and arterial smooth muscle cells in culture. It has now been established that LDL and HDL_C are specifically bound to the same high-affinity cell surface receptor site whereas the typical HDL are not. Considerable progress has been made this year in defining the chemical nature of the recognition site

on the lipoproteins responsible for binding to the receptor. The following conclusions, as reviewed in the progress reports, are now possible: 1) the HDL_C and LDL bind to the same receptor site, 2) the protein moieties of the lipoproteins determine the specificity for binding, 3) both the B apoprotein of LDL and the arginine-rich apoprotein of HDL_C are capable of binding to the receptor, 4) a similar positively charged region or structural sequence of the protein may be shared in common between the B and arginine-rich apoproteins, and 5) the arginyl residues are functionally significant residues in the lipoprotein recognition site.

Our detailed understanding of the cholesterol-induced lipoproteins of the various animal species has provided a strong basis for the investigation of the effects of cholesterol feeding in man. Studies are in progress to supplement the diet of men and women (20-45 years of age) with 4 to 6 eggs per day for 3-4 weeks and to characterize in detail the changes in the plasma lipoproteins. Results obtained in six subjects studied to date indicate that the equivalent of the HDL_C occurs in people after cholesterol (egg) feeding in a subfraction of the d=1.063-1.125 ultracentrifugal fraction. Furthermore, this lipoprotein appears following cholesterol feeding with or without an elevation of the plasma cholesterol level. This subfraction has characteristics similar to those described for the HDL_C of the lower species and binds to the cell surface receptors. These are potentially important results which may alter our interpretations of the role of high-density lipoprotein in the development of atherosclerosis.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do **NOT** use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02802 05 EA

PERIOD COVERED July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

The relationship of arterial intimal Evans blue dye concentration to surface reflectance and light absorption

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

PI: D.L. Fry Chief, Lab. of Exptl. Atherosclerosis EA NHLBI-IR

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Atherosclerosis

SECTION

INSTITUTE AND LOCATION

NHLBI-IR/NIH/Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.60	.10	.50

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 establish and validate the relationship between the concentration of Evans blue dye in the vascular interface and the absorption of red light.

879

PHS-6040
(Rev. 10-76)

Project Description

Objective: To establish and validate the relationship between the concentration of Evans blue dye in the vascular interface and the absorption of red light.

Methods Employed: A special reflectance measuring system was designed and constructed, such that opened vessel surfaces could be viewed under uniform illumination through a lens system on a large glass screen. A small aperature in the center of this screen led to the sensing surface of a photomultiplier tube. Appropriate light filters could be inserted in this light path as needed. The opened vessel surfaces were held in a specially designed servo-motor-driven tray submerged in saline, both to act as a metabolic support milieu for the vessel surface, as well as providing a nonreflecting optical coupling to the photo-sensing system. Various regions of the vessel surface could then be maneuvered by the operator under visual control such that a very small region on the vessel surface could be placed under the aperture in the screen. With a Wrattan 29 filter in the light path, it was possible to measure the amount of red light that is absorbed and reflected from the vessel surface. By staining various regions on the vessel surface to varying depths, it is possible to establish a monotonic relationship between the optical absorbance ρ (negative log of the ratio of light intensity from stained surface to that from adjacent unstained surface) of the stained surface and time (duration of exposure to EBD-albumin complex). A reflectance model based on the Lambert-Beer law of light absorption was evaluated to represent this relationship by fitting model parameters to data obtained as dye was eluted back out of the surface using an untagged albumin solution. The final model that was chosen implicitly predicts the optical absorption coefficient for EBD from these fitted parameters and also the change in vessel surface area that occurs with stretch of the wall. The accuracies of these implications were tested by measuring the EBD absorption coefficient directly in an Amino microphotometer and by measuring the correlation between measured change in wall stretch with those predicted from reflectance measurements.

Major Findings: It was found that the accumulation of EBD in the vascular intima bears an approximately logarithmic relationship to the amount of light absorbed as given by $M = k_{1}\rho + k_{3}\rho^{3}$, where M is the surface accumulation in nmoles/cm^2 and ρ is the optical absorbance. The constants k_1 and k_2 were found to be 3.92 and 0.80, respectively.

The validity of the model was established by noting that the predicted value of the absorption coefficient (from the fitted parameters) was 0.128 as compared to 0.126 for the directly measured value. The linear regression of the predicted wall stretch data on directly measured values had a slope of 0.99, a negligible intercept, and a correlation coefficient of 0.99. We conclude that the model represents the actual physical state with good precision.

Significance to Biomedical Research and the Program of the Institute: When Evans blue dye is injected intraveneously into an animal, virtually all of the dye becomes bound to serum albumin. Knowing the molar ratio of Evans blue dye to albumin permits the use of Evans blue dye as a measurable visual tag for albumin. Measurement of the amount of Evans blue dye in the intima thus can be used as a measure of transvascular macromolecular transport. We have shown that the atherosclerotic process is greatly enhanced in regions of increased EBD accumulation (increased transvascular transport of plasma proteins). The ability to quantify the topography of permeability in the opened vascular interface with this relatively simple reflectometric scanning technique opens many new areas in the study of the mechanisms of atherogenesis.

Proposed Course of the Project: This methodology is being applied to study of the topographic pattern of vascular permeability and its relation to the topography of atherosclerotic lesions in animals. The second major area of application will be to quantify permeability changes that occur with various altered hemodynamic events known also to be related to atherogenesis.

Publications

Fry, D.L. Aortic Evans blue dye accumulation: its measurement and interpretation. Am. J. Physiol. 232:H202-H222. 1977.

Fry, D.L. Hemodynamic forces in atherogenesis. Cerebrovascular Diseases. P. Steinberg, M.D., editor. New York. Raven Press. 1976. pp. 77-95.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02803 04 EA

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
The study of arterial transport processes in an in vitro support system

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

PI:	D.L. Fry	Chief, Lab. of Exptl. Atherosclerosis	EA NHLBI-IR
OTHER:	R.W. Mahley	Head, Comparative Atherosclerosis and Arterial Metabolism Section	EA NHLBI-IR

COOPERATING UNITS (if any)

NONE

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SL

INSTITUTE AND LOCATION
NHLBI-IR/NIH/Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.20	.20	1.00

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 study was to measure the concentration distribution of radiolabeled albumin across the deendothelialized aortic wall under conditions of positive and negative unidirectional as well as simultaneous positive and negative albumin flux under conditions approaching steady state transport.

882

PHS-6040

Project Description

Objective: The purpose of this study was to measure the concentration distribution of radiolabeled albumin across the deendothelialized aortic wall under conditions of positive and negative unidirectional as well as simultaneous positive and negative albumin flux under conditions approaching steady state transport.

Methods Employed: Aortas from dogs and swine were quickly removed under special conditions and placed in a specially designed device containing matching chambers which allowed the chemical milieu of the intimal and adventitial surfaces to be under experimental control. Radiolabeled albumin solutions ionically matched to plasma but having different concentrations of albumin were placed either in the intimal chamber or adventitial chamber or simultaneously in both chambers so that positive, negative, or simultaneous positive and negative albumin fluxes were imposed across the arterial wall. After 24 hours of exposure in a metabolically supported milieu, the radiolabeled reagents were aspirated and the tissue rapidly fixed in 3% glutaraldehyde. The tissues were then excised and sectioned on a freezing microtome for autoradiographic preparation. Additional specimens were removed for detailed histologic, histochemical, and electron microscopic examination. The distribution of developed silver across the arterial wall was quantified using a newly developed electron probe microanalytic technique (see progress report no. 02815).

Major Findings: It was found that the concentration distribution of radiolabeled albumin across the arterial wall could be approximated by 4 linear segments which, when compared to corresponding histologic sections, corresponded to 4 major zones of histologic structure. Thus, for purposes of diffusive transport, the arterial wall cannot be considered as a "homogeneous slab" but must be considered to consist of at least 4 contiguous zones each having approximately homogeneous characteristics. It was also shown that at each point across the wall the sum of the concentration for the positive and negative fluxes equalled the concentration for the tissue exposed to simultaneous positive and negative fluxes. When the concentration distributions were normalized to the concentration of radiolabeled albumin in the liquid milieu, it was found that the normalized distributions for varying concentrations of albumin were superimposable. This showed that the transport processes across the arterial tissue are insensitive to the concentration of albumin in the milieu or in the tissue which provides further evidence for the validity of the principle of superposition.

Significance to Biomedical Research and the Program of the Institute: This work describes for the first time a methodology that allows the quantitative determination of radiolabeled proteins across arterial tissue and presumably also is applicable to other tissue systems. Unlike many other polymer systems, the diffusive transport of albumin across arterial tissue appears to be independent of concentration or the direction of the flux which are extremely important facts in the conceptualization of transport processes in arterial tissue, i.e., the principles of superposition can be applied to

analysis of these processes. These data also establish values for the partition coefficient and relative magnitude of the diffusivity of arterial tissue across the wall as a function of its structural components. These data provide a point of departure in the design of further studies to determine the other parameters of macromolecular transport such as filtration coefficients, binding coefficients, chemical rate constants, and reflection coefficients. As these important parameters of transport become better defined, we shall then be able to place in proper perspective the role of transport processes in the genesis of arterial lipid deposition and reabsorption associated with atherogenesis.

Proposed Course of the Project: This methodology will now be extended to study similar processes in muscular arteries in normal animals as well as those in the process of developing atherosclerosis. They will also be extended to study the relative importance of convective processes in transport and their relationship to atherogenesis and regression.

Publications

None

884

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do **NOT** use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02805 03 EA

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

The simultaneous transport of ^{125}I albumin and EBD albumin into the arterial intimal medial space (new title)

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

PI:	D.L. Fry	Chief, Lab. of Exptl. Atherosclerosis	EA NHLBI-IR
OTHER:	R.W. Mahley	Head, Comparative Atherosclerosis and Arterial Metabolism Section	EA NHLBI-IR
	K.H. Weisgraber	Expert	EA NHLBI-IR

COOPERATING UNITS (if any)

NONE

LAB/BRANCH

Laboratory of Experimental Atherosclerosis

INSTITUTE AND LOCATION

NHLBI-IR/NIH/Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.20	.20	1.00

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 measure the simultaneous uptake of iodinated albumin and Evans blue dye complexed albumin by the normal and the deendothelialized aortic wall.

885

PHS-6040
(Rev. 10-76)

Project Description

Objective: The purpose of this project is to measure the simultaneous uptake of iodinated albumin and Evans blue dye complexed albumin by the normal and the deendothelialized aortic wall.

Methods Employed: A specially designed in vitro test system, described elsewhere, was used to control the flux of ^{125}Iodine tagged albumin (I-alb) and Evans blue dye tagged albumin (EBD-alb) into the arterial wall. This system permitted the arterial surface to be exposed to the tagged albumin under controlled conditions for various selected periods of time. Special experimental maneuvers were designed such that it was possible to study the uptake of albumin as a function of duration of exposure to the tagged albumin for the normal endothelial surface and the deendothelialized surface. The endothelial surface was removed by lightly brushing it with a serum-soaked camel hair brush. This surface was shown by scanning electron microscopy (as well as transmission electron microscopy) to have an absent endothelial cell layer but an intact basement membrane and internal elastica. The uptake of EBD-alb was quantified using a newly developed reflectance technique (see progress report no. 02802) and the uptake of I-alb by measurement of gamma activity. Certain ancillary studies were required to permit a precise quantitative comparison of these two uptakes. The most important of these was the measurement of the amount of radiolabeled albumin that was lost during the fixation process.

Major Findings: Fixation of arterial tissue with 3% glutaraldehyde phosphate buffered fixative was found to be the most efficient fixative of a variety that were tested. It was found that 90% of the fixation loss occurred during the first minute of fixation and that by 10 minutes glutaraldehyde had penetrated the arterial tissue sufficiently to immobilize all radiolabeled albumin to the fixed structural substances in the wall. The relationship of the uptake of EBD-alb and I-alb by the normal endothelialized arterial wall was found to be linear with time. However, it was found that the uptake of I-alb was nearly twice that for the EBD-alb. For the injured surface the relationship between the uptake of EBD-alb and I-alb was slightly curvilinear and the relationship with time markedly so, following an approximate "square root of time" law. In contrast to the uptake for the normal surface, the uptake of EBD-alb was about 4 times greater than for I-alb.

Significance to Biomedical Research and the Program of the Institute: The fixation loss data established glutaraldehyde as a fixative of choice for the quantitative study of the transport of radiolabeled albumin and presumably also other proteins across the arterial wall. These data indicate that the distribution of fixed radiolabeled protein should represent accurately the prefixation state except at the immediate interfacial regions. The uptake studies indicate that EBD may be used in place of radiolabeled albumin as a measure of the accessibility of the intimal medial space by albumin and presumably other plasma macromolecules. The predominance of dye uptake over iodine uptake by the injured surface implies significant binding of the dye by the arterial wall, and the inverse relationship for the uptake by the

normal surface implies a larger reflection coefficient for the EBD albumin complex as opposed to albumin itself. The curvilinear uptake as a function of time suggests that the uptake of albumin is controlled predominantly by diffusive forces in the injured case, and the linear temporal uptake by the normal surface implies a very large barrier to transport at the endothelial surface. Thus, apart from establishing for the first time in a quantitative fashion the efficiency of glutaraldehyde as a fixative for diffusable proteins, this work is of most significance in providing a new insight into the mechanics of macromolecular transport at the vascular interface.

Proposed Course of Project: These particular studies have been finished and provided a foundation and point of departure for the design of new studies to extend these observations to lipoprotein transport.

Publications

Fry, D.L., R.W. Mahley, K.H. Weisgraber, and S.Y. Oh. Simultaneous accumulation of Evans blue dye and albumin in the canine aortic wall. Am. J. Physiol. 233:H66-H79, 1977.

Fry, D.L. Aortic Evans blue dye accumulation: its measurement and interpretation. Am. J. Physiol. 232:H204-H222, 1977

Fry, D.L. Hemodynamic forces in atherogenesis. Cerebrovascular Diseases. P. Steinberg, ed. New York. Raven Press. pp. 77-95, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02806 04 EA

PERIOD COVERED
July 1, 1976 through September 20, 1977

TITLE OF PROJECT (80 characters or less)
Blood velocity profiles and hemodynamic stresses in the aorta and its major branches

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

PI:	D.J. Patel	Medical Officer	EA NHLBI-IR
OTHER:	H.B. Atabek	Professor, Catholic Univ. of America	
	S.C. Ling	Professor, Catholic Univ. of America	

COOPERATING UNITS (if any)
Mechanical Engineering Department and Department of Civil Engineering, The Catholic University of America, Washington, DC

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION
Vascular Physiology Section

INSTITUTE AND LOCATION
NHLBI-IR/NIH/Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.4	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)

It is the long-range purpose of this project to study the blood velocity profiles and hemodynamic stresses in the aorta and its major branches. The topics of present interest are: 1) a study of the geometry and elastic properties of the aorto-iliac junction to provide boundary conditions for detailed flow studies in this area; and 2) experimental validation of the nonlinear theory of pulse propagation to predict flow fields at more distal sites along the aorta from pressure-flow measurements made at a proximal site.

889

Project Description

Objective: 1) To study the geometry and elastic properties of the aorto-iliac junction in dogs, and 2) to test experimentally the nonlinear theory of pulse propagation for predicting flow fields at more distal sites along the aorta using pressure-flow measurements made proximal to the test site.

Methods Employed: To determine the geometry and the pressure-area relationship in the aorto-iliac junction area, a 16 mm cine camera equipped with a high-power telephoto lens was employed. Motion pictures of the pulsating arteries were taken. The arterial pressure was also monitored. The resulting information was used for determining the pressure-area relation. At the end of the filming, the animal was sacrificed and the region of interest was filled with a casting material under the mean value of the in vivo pressure. The hardened casts were taken out of the animal and sliced at regular intervals. Enlarged projections of these slices were used to determine the variations of shape and cross-sectional area along the aorta, at the junction and along the branches. In a few instances, the pressure-area relation was also studied in vitro following the in vivo studies. 2) The nonlinear theory of pulse propagation developed by Dr. Atabek was tested experimentally in a distensible silicon rubber model. Using the pressure gradient as input at the proximal end, the flow field at the distal end was computed and verified using measured values of pressure and center-line velocity.

Major Findings: 1) The cross-sectional area of the aorto-iliac junction changes from a circular cross section in the lower abdominal aorta to an oval form which flattens on the ventral side, eventually separating into 3 arteries with circular cross sections. 2) The aorto-iliac junction is more distensible near the abdominal aorta, becoming stiffer as one approaches the peripheral arteries. 3) The nonlinear theory of pulse propagation predicted the distal flow fields accurately in a distensible silicon rubber tube.

Significance to Biomedical Research and the Program of the Institute: Hemodynamic stresses are thought to play a central role in the etiology of early atherosclerosis. In order to study this role quantitatively, we need to measure, in vivo, flow fields in the critical areas of the circulatory system. The present study of the aorto-iliac function will provide boundary conditions for flow studies in this region, and the nonlinear theory of pulse propagation could predict flow fields at more distal inaccessible sites from measurements made at a proximal site. These represent a modest step toward our goal to measure or compute flow fields in the critical areas of the circulatory system.

Proposed Course of the Project: 1) Two manuscripts covering this phase of the project are in preparation; 2) We plan to test the nonlinear theory of pulse propagation in vivo in the dog aorta.

Publications

Hung, T., R. Skalak, G. Bugliarello, Y.K. Liu, D.J. Patel, and M.S. Albin. Perspectives in biomechanics research and education for the next decade. J. of Engineering Mechanics Division. ASCE. In press.

Vaishnav, R.N., H.B. Atabek, and D.J. Patel. Properties of the intimal layer and adjacent flow. J. of Engineering Mechanics Division, ASCE. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02807 04 EA

PERIOD COVERED July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Vascular mechanics: local properties of the intimal layer of large arteries (new title)

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

PI:	D.J. Patel	Medical Officer	EA NHLBI-IR
OTHER:	R.N. Vaishnav	Professor, Catholic Univ. of America	

COOPERATING UNITS (if any)
Department of Civil Engineering, The Catholic University of America, Washington, DC

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION
Vascular Physiology Section

INSTITUTE AND LOCATION
NHLBI-IR/NIH/Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.4	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 purpose of this project was to study the local viscoelastic properties of the intimal layer of large arteries with a view towards understanding their role in atherogenesis. The strength of the endothelium was studied by exposing the endothelial surface to a saline jet of known strength and quantifying the resulting damage to the endothelial cells. The compliance of the intimal layer was studied by exposing the surface to a microindentor and measuring the indentation of the intimal surface as a function of applied force. These studies will shed added light on the role of mechanical factors in the pathogenesis of atherosclerosis.

891

Project Description

Objective: To study the local viscoelastic properties of the intimal layer of large arteries with a view towards understanding their role in atherogenesis.

Methods Employed: Two methods were used to study local mechanical properties of the intimal layer of excised segments of the canine thoracic aorta stretched to in vivo dimensions. In one set of experiments, designed to study the strength of the endothelium, the endothelial surface was exposed to saline jets of known strength acting normal to the surface; the damaged surface was then stained with Evans blue dye and examined under a microscope. In the second set of experiments, designed to study the compliance of the intimal layer, the surface hardness was determined from point to point by a microindentor which applied a known force to the surface and measured the resulting indentation.

After preliminary work on the two methods was completed, attention was focused in two directions. For the first method, we concentrated on calibration of the jet to determine the shear stress on the endothelium as a function of the radial distance from the epicenter of the jet, using the hot-film anemometric technique on the prototype jet as well as on scaled up models. For the second method, we redesigned (with the help of Mr. F. Plowman) the microindentor using an air-bearing as the pivot instead of a knife edge.

Major Findings: For the jet calibration, we have obtained curves of the general shape as would be expected for qualitative considerations, but are in the process of further refining the method so that the quantitative results are dependable. Our objective is to have a master curve expressing shear stress as a function of the radial distance.

The mechanical aspects of the new microindentor apparatus have been tested and found satisfactory.

Significance to Biomedical Research and the Program of the Institute: There is considerable evidence that hemodynamic events and local tissue factors play a significant role in the development of atherosclerosis. One of the ways in which this role is manifested is the effect of high shearing stresses in increasing the permeability of the endothelial surface to macromolecules. The saline jet studies seek to quantify the strength of this endothelial surface and thus are significant in our understanding of the atherosclerotic processes. There is also evidence pointing to the possibility that when exposed to chronic high shear stresses such as those experienced at intercostal orifices, branches, etc., the tissue responds physiologically by undergoing defensive structural changes in the intimal layer. Histologically, such changes have been observed, for example, in formation of intimal pads at intercostal orifices. The microindentation method provides a mechanical method for studying this phenomenon, and hence is significant in the study of atherogenesis.

Proposed Course of the Project: 1) The master calibration curve will be used to translate the diameters for the jet lesions obtained so far into equivalent shear strength values and then use the method to study systematically the local yield strength of the endothelium in critical areas of the circulatory system. 2) The microindentation apparatus will be used to map the compliance of the intimal surface of the aorta with a view towards correlating these maps with the corresponding maps obtained by Dr. Fry in his permeability studies.

Publications

Patel, D.J., R.N. Vaishnav. Mechanical properties of arteries. Cardiovascular Flow Dynamics and Measurements. edited by Ned H. Hwang and Nils A. Normann. University Park Press. Baltimore. 1977. pp. 439-472.

Patel, D.J., R.N. Vaishnav, H.B. Atabek, F. Plowman. Measurement of endothelial strength using saline jets. Microcirculation. 1:70-80. 1976.

Vaishnav, R.N., J.T. Young, D.J. Patel. Nonlinear viscoelastic theory for large blood vessels. Proceedings of the First International Congress on Cardiovascular System Dynamics. Valley Forge, PA (Summer 1977) (In press).

Young, J.T., R.N. Vaishnav, D.J. Patel. Nonlinear anisotropic viscoelastic properties of canine arterial segments. J. of Biomechanics. 1977 (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02808 04 EA

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Trial of psychophysiologic techniques for the amelioration of hypertension

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

PI:	D.J. Patel	Medical Officer	EA NHLBI-IR
	B.L. Frankel	Staff Investigator	N SN
	D. Horwitz	Senior Investigator	HE NHLBI
OTHER:	W.T. Friedewald	Chief, Clin. Trials Br.	CT NHLBI
	E. Freis	Senior Investigator, VA Hospital	

COOPERATING UNITS (if any)
Veterans Administration Hospital

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SE
Vascular Physiology Section

INSTITUTE AND LOCATION
NHLBI-IR/NIH/Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	0.2	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 study was intended to determine whether a combination of psychophysiologic techniques including biofeedback and autogenic exercises could produce a sustained, therapeutically useful reduction in blood pressure in patients with essential hypertension. The results suggest that such a combination is not more clinically effective than a placebo procedure; both of these approaches, however, appear to be more therapeutic than no treatment.

894

Project Description

Objective: Studies reporting that elevated blood pressure (BP) can be lowered by psychophysiologic techniques such as biofeedback, autogenic exercises, hypnosis, and transcendental meditation have not provided definitive information on how long the effects were sustained at clinically meaningful lower levels and usually were not adequately controlled. The present study was designed to determine whether a combination of psychophysiologic techniques could produce a sustained, therapeutically useful reduction in BP in patients with essential hypertension.

Methods Employed: The study group consisted of 22 patients with uncomplicated essential hypertension who showed mean diastolic BP levels between 90 and 105 while supine. Patients underwent an initial eight-week period of evaluation during which BP was taken at least once a week by a nurse-observer blind to the patient's experimental status throughout the study. The mean value of the BP measurements of the final 6 of these 8 weeks were used in assessing pre-treatment ("baseline") BP levels. Thereafter, patients entered a sixteen-week study period during which the weekly BP determinations continued. Means of BP data recorded during the final 6 weeks of this sixteen-week period were compared to the means of baseline measures to evaluate overall therapeutic effects.

First Phase Studies

Patients were randomly allocated to one of three groups:

1. Active Treatment (AT) Group (N=7). In 20 laboratory sessions, AT patients were trained in the use of electromyographic and/or skin temperature biofeedback and diastolic BP feedback. They also learned autogenic exercises and relaxation training. During laboratory training sessions, pulse, BP, finger temperature, and frontalis muscle activity were monitored noninvasively. These patients also pursued a monitored daily home practice program to reinforce and generalize laboratory learning.

2. Pseudo-Treatment (PT) Group (N=7). In each of 20 laboratory sessions, these patients received only noncontingent or pseudodiastolic BP feedback arranged to convey a sense of success to them. While laboratory instrumentation and physiologic monitoring was the same for this group as for the AT group, they did not practice any techniques at home.

3. No-Treatment Control (NTC) Group (N=8). After completing the eight-week baseline period, NTC patients participated in a minimal program consisting of weekly BP determinations by the nurse-observer for 16 more weeks.

Second Phase Studies

PT and NTC patients were offered the opportunity to participate in the AT protocol after completing their initial sixteen-week period, each patient serving as his own control. Patients in any of the three groups having a

decrease in mean diastolic BP to either less than 90 mm Hg or to a level at least 10% below baseline values were studied for an additional 2 months to determine if this fall was sustained.

Major Findings: Of the 7 AT patients, two showed clinically unequivocal decreases in post-AT supine diastolic BP (from 95.8 to 87.8 mm Hg in one; from 90.5 to 86.5 mm Hg in the other). One AT patient had a modest decrease in diastolic BP (from 93.7 to 90.5 mm Hg). The other 4 AT patients were treatment failures. Of the 7 PT patients, one showed a modest decrease in the mean post-PT diastolic BP (from 104.3 to 100.0 mm Hg); he, thereafter, participated in active treatment for 16 more weeks and had a further comparable decrease (from 100 to 95.8 mm Hg). Of the 4 PT patients having no post-PT changes in BP, 3 continued with the AT protocol; of these 3, two had no post-AT changes in BP, while one had a clinically meaningful BP fall (from 94.7 to 86.7 mm Hg). Seven of the 8 NTC patients showed no BP decreases while in one it fell from 97.0 to 93.0 mm Hg. This patient's BP decreased further from 93.0 to 90.0 mm Hg after completing her AT participation.

There did not appear to be any clear-cut psychological or physiological patterns of predictive value in those patients who responded to active or pseudo-treatment.

Significance to Biomedical Research and the Program of the Institute: These findings suggest that the use of a combination of elaborate psychophysiologic techniques in essential hypertension is not clearly more clinically effective than a placebo procedure. However, since both techniques appear to be more effective than no treatment, certain factors common to both (e.g., regular periods of prolonged relaxation) may be of significant clinical value to some patients with essential hypertension. Further studies might focus more on delineating these patient characteristics predictive of clinical responsiveness.

Proposed Course of the Project: This project has been completed; two manuscripts have been prepared for publication.

Publications

None

896

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02809 03 EA

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Quantitation of the apolipoproteins in plasma by two-dimensional immunoelectrophoresis and radioimmunoassay (new title)

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

PI:	R.W. Mahley	Head, Comparative Atherosclerosis and Arterial Metabolism Section	EA NHLBI-IR
OTHER:	K.S. Holcombe	Chemist	EA NHLBI-IR

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SI
Comparative Atherosclerosis and Arterial Metabolism Section

INSTITUTE AND LOCATION
NHLBI-IR/NIH/Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.75	.25	.50

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 is the purpose of this project to develop a relatively simple and rapid quantitative immunochemical method for apolipoprotein analysis. The method has been validated using lipoproteins from control and cholesterol-fed rats for the arginine-rich and A-I apoproteins. It is presently being extended to the analysis of various swine and human apoproteins.

897

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To determine changes in the distribution of apolipoproteins in the plasma of control and cholesterol-fed animals and man. Also, to monitor changes in cholesterol-induced hyperlipoproteinemia following hypolipidemic drug therapy.

Methods Employed: Monospecific antisera has been prepared to the "arginine-rich," A-I, and B-apoproteins of the rat, dog, miniature swine, and human lipoproteins. Laurell's two-dimensional quantitative immunoelectrophoretic procedures as revised by Versey and Davis has been modified for lipoprotein apoprotein quantitation. Our modification of the method, which includes delipidation of samples by triton, has overcome a major difficulty of apoprotein quantitation, i.e., failure to analyze hypertriglyceridemic plasma because of the presence of large, poorly migrating lipoproteins. In addition to allowing quantitation of all plasma samples, delipidation also reduced the possibility of masked antigenicity.

The rat plasma lipoproteins are the principal lipoproteins under investigation at this time. The plasma lipoproteins of rats are fractionated by ultracentrifugation into four major density classes, d<1.006 (VLDL); 1.006-1.019 (intermediate); 1.019-1.063 (LDL and HDL_1 or HDL_c) and 1.063-1.21 (HDL_2). The quantitative distribution of the various apoproteins is determined in each ultracentrifugal fraction for control chow fed animals vs. rats on various hypercholesterolemic diets. The hypercholesterolemic diets contain lard and cholesterol plus a bile acid (either cholic acid or taurocholate). In addition, some of the animals also receive propylthiouracil (PTU). The alterations in lipoprotein metabolism following administration of hypolipidemic drugs is determined by analysis of changes in the apolipoprotein distribution.

Radioimmunoassays are also being established to provide greater sensitivity and to facilitate the analysis of a large number of samples of human plasma and plasma lipoprotein fractions. Delipidated samples are incubated in the presence of pure ^{125}I-apoprotein for varying periods of time. By use of the second antibody technique, the amount of displacement of the ^{125}I-apoprotein by the sample is determined. The assays for the human A-I and arginine-rich apoproteins are being validated.

Major Findings: A modification of the two-dimensional immunoelectrophoretic technique has been validated for the assay of rat arginine-rich and A-I apoproteins. It has demonstrated that the cholesterol feeding results in a two-fold increase in the arginine-rich apoprotein from a central value of 29 mg/100 ml to 58 mg/100 ml. The increase occurs in the d<1.006 (B-VLDL), intermediate lipoproteins, and HDL_c of the cholesterol-fed rats. An additional major finding is that a very substantial quantity of the arginine-rich apoprotein (>50%) occurs in the d>1.21 following ultracentrifugation. It remains to be determined as to how much of the arginine-rich apoprotein is actually free in the plasma. The immunochemical techniques will be used to quantitate the apolipoprotein in support of the projects described under lipoprotein characterization and tissue culture.

Significance to Biomedical Research and the Program of the Institute: A relatively simple and rapid quantitative method for apolipoprotein analysis provides a useful tool to monitor changes in plasma lipoproteins induced by dietary manipulations and drug therapy. These studies will provide insight into changes in lipoprotein metabolism associated with the development of atherosclerosis following cholesterol feeding or the reversal of atherosclerosis following drug therapy.

Proposed Course of the Project: The project will continue along the lines indicated above. The methods will be extended to the analysis of human lipoprotein changes induced by diet and drug therapy.

Publications

Mahley, R.W., and K.S. Holcombe. Alterations of the plasma lipoprotein and apoproteins following cholesterol feeding in the rat. J. Lipid Res. 18:314, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02810 06 EA

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Hyperlipoproteinemia and atherosclerosis: changes in plasma lipoproteins and apolipoproteins induced by cholesterol feeding

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

PI:	R.W. Mahley	Head, Comparative Atherosclerosis and Arterial Metabolism Section	EA NHLBI-IR
	K.W. Weisgraber	Expert	EA NHLBI-IR
	D.L. Fry	Chief, Laboratory of Experimental Atherosclerosis	EA NHLBI-IR
OTHER:	K.S. Holcombe	Chemist	EA NHLBI-IR

COOPERATING UNITS (if any)

Meloy Laboratories, Springfield, VA - NHLBI Contract No. N01 HI 3 2926

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Laboratory of Experimental Atherosclerosis

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.25	1.00	1.25

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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)

It is the purpose of this study to correlate the changes in plasma lipoproteins in animals fed cholesterol-rich diets with the development of accelerated atherosclerosis. The characterization of the lipoproteins induced by cholesterol feeding in dogs, swine, monkeys, rats, and rabbits has established that various species have a similar metabolic response to increased dietary cholesterol. The hyperlipoproteinemia has certain consistent features which include the occurrence of beta-very low density lipoproteins, an increase in low density lipoproteins, and the appearance of a unique lipoprotein, the HDL_C. The occurrence in increased concentration of a specific apoprotein (arginine-rich apoproteins) with all these cholesterol induced lipoproteins suggests an important role for this protein in cholesterol metabolism and possibly in accelerated heart disease.

900

Project Description

Objectives: 1) To characterize the lipoproteins and apoproteins from control dogs, miniature swine, Patas monkeys, rats, and rabbits, and to compare these to changes induced by cholesterol feeding. 2) To correlate the type of hyperlipoproteinemia with the type, distribution and degree of atherosclerosis.

Methods Employed: The various animal species, including dogs, swine, monkeys, rats, and rabbits, were fed diets which contain 0.5 to 2.0% cholesterol, as described previously. Isolation of the plasma lipoproteins was accomplished by the combination of ultracentrifugation and Geon-Pevikon block electrophoresis. The purified lipoproteins were characterized with respect to electrophoretic mobility, immunochemical reactivity, size by electron microscopy, chemical composition and apoproteins. The apoproteins were isolated and purified by Sephadex and DEAE column chromatography. Analyses of the apoproteins included amino acid analysis, N- and C-terminal amino acids, and molecular weights.

Major Findings: Dogs, miniature swine, rats, rabbits, and Patas monkeys fed high-cholesterol diets had a similar lipoprotein response which was associated with the development of atherosclerosis. Animals on a low cholesterol diet served as controls. The characteristics of the hyperlipoproteinemia associated with atherosclerosis were as follows: 1) The beta-VLDL become prominent lipoproteins. The B-VLDL are beta-migrating lipoproteins in the d<1.006 fraction which resemble the beta-VLDL of human Type III hyperlipoproteinemia, particularly with respect to the prominence of the "arginine-rich" apoprotein. 2) LDL and the intermediate lipoproteins (IDL) are present in increased concentrations and are variably enriched in the "arginine-rich apoprotein. These lipoproteins and the beta-VLDL may represent remnants of intestinal lipoproteins induced to transport the dietary lipid. 3) A unique class of lipoproteins, which we have called HDL_C, are a consistent feature following cholesterol feeding. These lipoproteins are cholesterol-rich and contain the "arginine-rich" apoprotein and A-1. They lack the B-apoprotein. The HDL_C are important regulators of sterol synthesis in aortic smooth muscle cells and fibroblasts (see project no. 02813).

Characterization of the "arginine-rich" apoprotein suggest homology of this protein among the species. They have a similar amino acid analysis and contain 12 moles% arginine. The "arginine-rich" apoprotein appears to play an essential role in cholesterol transport between lipoproteins and possibly between lipoproteins and the aortic wall (atherosclerotic lesion).

Significance to Biomedical Research and the Program of the Institute: Characterization of cholesterol induced hyperlipoproteinemia and development of animal models resembling human disease will enable us to better understand human lipoprotein metabolism. In addition, these studies are designed to correlate the type of hyperlipoproteinemia with the type distribution and degree of experimentally induced atherosclerosis.

Proposed Course of the Project: The project will continue along the lines indicated above with an extension in the detailed characterization of human lipoproteins.

Publications

Mahley, R.W., K.H. Weisgraber, T. Innerarity, and H.B. Brewer, Jr. Characterization of the plasma lipoproteins and apoproteins of the Erythrocebus Patas monkey. Biochemistry. 15:1928, 1976.

Mahley, R.W., K.H. Weisgraber, and T. Innerarity. Atherogenic hyperlipoproteinemia induced by cholesterol feeding in the Patas monkey. Biochemistry. 15:2979, 1976.

Mahley, R.W., T.L. Innerarity, K.H. Weisgraber, and D.L. Fry. Canine hyperlipoproteinemia and atherosclerosis: Accumulation of lipid by aortic medial cells in vivo and in vitro. Am. J. Path. 87:205, 1977.

Tall, A.R., D. Atkinson, D.M. Small, and R.W. Mahley. Characterization of the lipoproteins of atherosclerotic swine. J. Biol. Chem. In press.

Mahley, R.W. Alterations in plasma lipoproteins induced by cholesterol feeding in animals including man. In: The Physiology of Lipids and Lipoproteins in Health and Disease. edited by J. Dietschy. In press.

Weisgraber, K.H., R.W. Mahley, and G. Assmann. The rat arginine-rich apoprotein and its redistribution following injection of iodinated lipoproteins into normal and hypercholesterolemic rats. Atherosclerosis. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02811 06 EA

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Aortic metabolism of plasma lipoproteins

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

PI: R.W. Mahley	Head, Comparative Atherosclerosis and Arterial Metabolism Section	EA NHLBI-IR
D.L. Fry	Chief, Laboratory of Experimental Atherosclerosis	EA NHLBI-IR
K.H.Weisgraber	Expert	EA NHLBI-IR

COOPERATING UNITS (if any)

None

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NHLBI-IR/NIH/Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	.75	.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)

It is the purpose of this study to determine which plasma lipoproteins are transported across the aortic endothelium, their mode of transport, and their fate in the aortic wall. Methodology for the in vitro maintenance of the aorta for periods of several hours has been developed.

903

Project Description

Observations: To determine 1) which classes of plasma lipoproteins are involved in aortic transport; 2) whether these lipoproteins are transported as intact macromolecules or hydrolyzed at the surface and components transported separately; 3) the fate of the lipoprotein components metabolized by the aorta.

Methods Employed: The in vitro transport method (described by Dr. D.L. Fry) is used to study aortic endothelial transport of the plasma lipoproteins under controlled conditions. Presently, the dog is being used as the experimental model; but we will soon extend this to the miniature swine. The components of canine plasma lipoproteins (VLDL, LDL, HDL_1 and HDL_2) are labeled with various radioisotopes. Initially, we attempted to use ^{125}I as our protein tag. After exhaustive studies we conclude that it is impossible to limit the ^{125}I to the protein moieties, and variable amounts of lipids are labeled. The ^{125}I lipid label is unstable. We are now able to label the protein moieties of the canine lipoproteins with ^{35}S-methionine in vivo. At the same time, we are able to label the phospholipids with ^{32}P-orthophosphate. ^{14}C-cholesteryl-esters and ^{3}H-free cholesterol moieties of these lipoproteins are labeled in vitro by the exchange method by Avigan. Uptake of label and the metabolism of the lipoproteins are followed by analysis of changes in the incubation media, examination of the aorta by direct isotope counting following oxygen combustion and by light and electron microscopic autoradiography.

Major Findings: Preliminary findings indicate the feasibility of this approach to the study of aortic endothelial transport and metabolism of plasma lipoprotein. Methodologic problems and validation of techniques continues to be a major component of this project. In addition to validation of the in vitro technique (described in a separate project report by Dr. D.L. Fry), methodology for the quantitation of four separate radioisotopes (^{14}C, ^{3}H, ^{35}S, and ^{32}P) has been established. The labeled plasma lipoproteins or a dried portion of the aorta following an in vitro transport study are placed in an oxygen combustion flask and ignited. ^{35}S-methionine which is the protein tag and ^{32}P which is the phospholipid tag are converted to inorganic sulfate and phosphate, respectively. These isotopes remain in the flask and are quantitated together by standard double label liquid scintillation counting. The flask is heated to drive off the tritium in the form of $^{3}H_2O$ and ^{14}C in the form of $^{14}CO_2$. The $^{14}CO_2$ is collected by bubbling the gas through a base converting it to an insoluble carbonate and the $^{3}H_2O$ is collected on a condenser in an ice bath. Isotope recovery is greater than 90% and modifications are being made to increase the efficiency of the method.

Significance to Biomedical Research and the Program of the Institute: It is agreed by most that 1) cholesterol within atheromata is derived largely from plasma lipoproteins and that 2) plasma lipoproteins can be detected within the same lesions. However, the mode of transport and the quantitative significance of lipoproteins in the lesions are far from clear. Serious questions remain as to whether the lipoproteins cross the endothelial surface

intact or whether they are hydrolyzed at the surface with only some of the components entering the tissue. Our in vitro approach to this problem should shed light on this most difficult problem of atherosclerosis research as well as add to our knowledge of lipoprotein metabolism.

Proposed Course of the Project: The project will be continued along the lines indicated above. It will also be extended to the miniature swine and nonhuman primates for comparative studies.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02812 08 EA

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Animal models for study of atherosclerosis

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

PI:	R.W. Mahley	Head, Comparative Atherosclerosis and Arterial Metabolism Section	EA NHLBI-IR
	D.L. Fry	Chief, Laboratory of Experimental Atherosclerosis	EA NHLBI-IR
OTHER:	J.E. Pierce	Chief, Section on Laboratory Animal Medicine and Surgery	OD NHLBI-IR
	J.M. Phillips	Chief, Unit on Lab. Animal Med. & Care	M-IR
	D.K. Johnson	Chief, Veterinary Medicine and Surgery Section	VR DRS
	R.M. Jaffe	Staff Physician	CP CC
	R.L. Killens	Chief, Comparative Medicine Unit	VR DRS
	V.J. Ferrans	Chief, Ultrastructure Section	PA NHLBI-IR

COOPERATING UNITS (if any)
Veterinary Resources Branch, Div. of Research Services; Clinical Pathology, Clinical Center; and Colorado State University (Res. Contract #N01 HI 4 2903); Comparative Medicine Unit, Division of Research Services

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION
Comparative Atherosclerosis and Arterial Metabolism Section

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NHLBI-IR/NIH/Bethesda, MD 20014

TOTAL MANYEARS: 3.0	PROFESSIONAL: 2.0	OTHER: 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)

It is the purpose of this study to establish the proper dietary and metabolic conditions necessary to induce experimental atherosclerosis in various species which has characteristics similar to that of man.

In three species, the dog, miniature swine, and Patas monkey, this has been accomplished. The source of dietary fat has been found to have a profound effect in the type, distribution, and severity of the atherosclerosis. The atherosclerosis produced by diets containing beef tallow, in addition to being severe, is associated with gross arterial thrombosis and occlusive vascular disease.

PHS-6040
(Rev. 10-76)

Project Description

Objective: To determine the suitability of a variety of animals as models for atherosclerosis as compared to the human disease.

Methods Employed: The animal models which have been studied in varying detail are the Patas monkey, miniature pig, dog, rabbit, and rat. The experimental conditions under which the pathologic processes in these animals can be made to resemble those in man have been detailed in previous project reports. Briefly, the disease can be induced in the rabbit, pig, and monkey by feeding diets high in cholesterol and fat (lard); whereas disease can be induced in the dog and rat to a comparable extent only if hypothroidism is also induced. In dogs the types of dietary fats are varied and included cottonseed oil, pork lard, beef tallow, safflower oil, and peanut oil.

Blood chemistries, which includes detailed lipoprotein studies, are monitored during the experimental period. At termination each animal is examined in detail using the standardized necropsy procedures as described previously. Topographic distribution and histologic characteristics of aortic, coronary, and peripheral arteries are compared to human atherosclerosis. The comparative human material is derived from young adults dying tramatic deaths, unselected hospital cases, and patients with documented types of hyperlipoproteinemia.

Major Findings: The animal colony studies in progress for several years continue to provide the basis for our understanding of several key questions in the field of lipoprotein metabolism and atherosclerosis. One of the strengths of our program has been the capability to compare the response of atherosclerosis-resistant (dog and rat) species with the susceptible (swine and monkey) species and to relate these findings to human disease. The approach has provided fundamental, basic information in several areas as follows:

1. Lipoprotein Metabolism. Certain consistent features of cholesterol-induced hyperlipoproteinemia in the various species have been described including the occurrence of a previously unrecognized class of plasma lipoproteins. The detailed understanding of the types of changes induced by cholesterol feeding in the animals (dogs, swine, rats, rabbits, and monkeys) has provided a background upon which to study the changes which occur with cholesterol feeding in man. A fundamental observation which is being actively studied in our laboratory is that the consumption of a cholesterol-rich diet by man, with or without an elevation of plasma cholesterol, alters the plasma lipoproteins in ways which resemble the changes observed in the lower species. In the animal studies such changes are associated with accelerated atherosclerosis.

2. Regulation of Cholesterol Metabolism in Arterial Smooth Muscle Cells and Fibroblasts by Plasma Lipoproteins. A recent contribution of our laboratory, which was provided by the uniqueness of the cholesterol-induced lipoproteins of dogs and swine, has been the identification of the determinants responsible for lipoprotein binding to specific cell surface receptors.

It is now established that the protein moieties of the lipoproteins are responsible for specific binding, that the B and arginine-rich apoproteins are responsible for the receptor binding, and that arginyl residues of the proteins are functionally important in the lipoprotein recognition site. In addition, the regulation of the rate and extent of intracellular deposition of cholesterol esters by arterial cells appears to be controlled by the type of lipoprotein which interacts with the receptor.

3. Role of Dietary Fat in Determining the Severity of Atherosclerosis and the Associated Complications of Thromboatherosclerosis. Dogs fed a high-cholesterol diet associated with either saturated or unsaturated fats develop atherosclerosis which differs in both distribution and severity. Diets containing saturated fat result in more severe atherosclerosis and a significant increase in the occurrence of thrombosis and the complications of thrombo-atherosclerosis (visceral organ infarction). Thromboatherosclerosis in dogs (and more recently in swine) on a high saturated fat-cholesterol diet has been correlated with platelet hypersensitivity to aggregation and release. These models provide for the first time an experimental approach by which to determine whether the role of the platelet in atherosclerosis is primary or secondary. In addition, these well-characterized models provide the opportunity to determine experimentally the factors which alter the course of the disease process. The animal models provide the only approach to many of the questions which need to be explored if we are to understand the role of diet, trace elements and vitamins, plasma lipoprotein levels, and hemostatic function in the pathogenesis of atherosclerosis. Many of the advances we have made relate directly to observations made first in the animals and then extended to man. Our success, as documented in the publications of the last year, clearly demonstrates our dependence upon the availability of high-quality animal holding facilities, dedicated animal handlers, and qualified veterinarian support.

Significance to Biomedical Research and the Program of the Institute: The "atherosclerotic process" is, in fact, an ensemble of processes occurring at the cellular, physicochemical, biochemical, and biophysical level in the arterial intima. The purpose of this program is to identify as many of these fundamental processes as possible, establish which are relevant to those in man, and study these in great detail in the animal model, wherein the pertinent variables can be measured or controlled with a rigor not possible in man.

A clear definition of the role of diet in the development of atherosclerosis in man is of utmost importance.

Proposed Course of the Project: The pursuit of the above objectives will continue with our NIH collaboration.

Publications

Mahley, R.W., A.W. Nelson, V.J. Ferrans, and D.L. Fry. Thrombosis in association with atherosclerosis induced by dietary perturbations in dogs. Science. 192:1139, 1976.

Mahley, R.W. Lipoproteins and arterial smooth muscle cells: Regulation of cellular metabolism by swine lipoproteins. International Workshop Conference on Atherosclerosis, London, Canada. Pleum Press. 1975. In press.

Mahley, R.W. Alterations in plasma lipoproteins induced by cholesterol feeding in animals including man. In. The Physiology of Lipids and Lipoproteins in Health and Disease. edited by J. Dietschy. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02813 05 EA

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Tissue culture studies of aortic smooth muscle cells and skin fibroblasts: cell growth and metabolism in response to incubation with various lipoprotein classes

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

PI:	T.P. Bersot	Clinical Associate	EA NHLBI-IR
OTHER:	R.W. Mahley	Head, Comparative Atherosclerosis and Arterial Metabolism Section	EA NHLBI-IR
	D.L. Fry	Chief, Laboratory of Experimental Atherosclerosis	EA NHLBI-IR

COOPERATING UNITS (if any)
Meloy Laboratories, Springfield, VA - NHLBI Contract No. N01 HI 3 2926

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Laboratory of Experimental Atherosclerosis

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INSTITUTE AND LOCATION
NHLBI-IR/NIH/Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.00	1.00	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)

These studies have continued to focus on the mechanism by which plasma lipoproteins deliver lipids to cultured cells. Using high density lipoproteins obtained from humans with abetalipoproteinemia, we have shown that apolipoprotein B is not necessary for the binding of human lipoproteins to cells in culture. Heparin precipitation has also been found to correlate with the extent of lipoprotein binding in that the lipoproteins which are precipitated most readily by heparin are also bound and internalized most avidly to cells in culture.

910

PHS-6040
(Rev. 10-76)

Project Description

Objectives: 1) To study the effect of various lipoprotein classes upon aortic smooth muscle cell proliferation. 2) To study the effects of various lipoprotein classes upon smooth muscle cell and skin fibroblasts cholesterol metabolism. 3) To establish strains of swine and canine endothelial cells in culture.

Methods Employed: 1) Standard techniques were used in determining cell proliferation in response to various classes of lipoproteins. 2) Electron microscopy was used to establish that cells were similar to smooth muscle and endothelial cells as reported in the literature. 3) Cellular cholesterol synthesis was assed by measuring the incorporation of [I-^{14}C] acetate into cholesterol and cholesteryl esters. 4) Binding, uptake, and degradation of iodinated lipoproteins were measured by standard techniques.

Major Findings: 1) The ability of a lipoprotein to be bound, internalized, and degraded by normal fibroblasts in culture was found to correlate directly with the extent of heparin precipitibility of the lipoprotein. Only lipoproteins containing apoprotein B or the arginine-rich apoprotein were heparin precepitible and these lipoproteins were the only ones capable of binding to the high affinity binding site. 2) Low density and high density lipoproteins obtained from patients with abetalipoproteinemia were found to bind to high affinity binding sites of normal fibroblasts despite absence of the B-apoprotein. 3) The high density lipoprotein fraction that binds with high affinity to fibroblasts does not transfer the same amount of cholesterol into cells as does LDL when the cells are incubated at equivalent protein or cholesterol concentrations.

Significance to Biomedical Research and the Program of the Institute: Heparin precipitation has been useful in identifying lipoproteins capable of binding to high affinity sites of cells in culture. This has been particularly helpful in isolating the subfractions of high density lipoproteins which bind to cells. The binding of abetalipoproteinemic patients' HDL and LDL extends to man the observation that apo B is not necessary for binding human lipoproteins to cells in culture. The fact that ARP-containing HDL can compete with LDL for binding and uptake yet not increase cellular cholesterol content indicates that ARP-containing lipoproteins may inhibit the entry of LDL cholesterol into the cell.

Proposed Course of the Project: Study of interaction of ARP-containing lipoproteins with cells in culture will continue. Emphasis will be placed upon how HDL containing ARP influences cellular cholesterol content.

Publications

Mahley, R.W., and T.L. Innerarity. Interaction of canine and swine lipoproteins with the low density lipoprotein receptor of fibroblasts as correlated with heparin/manganese precipitability. J. Biol. Chem. 252:3980, 1977.

Mahley, R.W., T.L. Innerarity, R.E. Pitas, K.H. Weisgraber, J.H. Brown, and E. Gross. Inhibition of lipoprotein binding to cell surface receptors of fibroblasts following selective modification of arginyl residues in arginine-rich and B apoproteins. J. Biol. Chem. In press.

Brown, B.G., R.W. Mahley, and G. Assmann. Swine aortic smooth muscle in tissue culture. Some effects of purified swine lipoproteins on cell growth and morphology. Circ. Res. 39:415, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02814 02 EA

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Topographic analyses of endothelial surface permeability and atherosclerosis

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

PI:	D.L. Fry	Chief, Laboratory of Exptl. Atherosclerosis	EA NHLBI-IR
OTHER:	R.W. Mahley	Head, Comparative Atherosclerosis and Arterial Metabolism Section	EA NHLBI-IR
	D.K. Johnson	Chief, Veterinary Medicine and Surgery Section	VR DRS
	J.E. Pierce	Chief, Section on Laboratory Animal Medicine and Surgery	OD NHLBI-IR
	J.M. Phillips	Senior Veterinary Officer	OD NHLBI-IR
	R.L. Killens	Chief, Comparative Medicine Unit	VR DRS

COOPERATING UNITS (if any)
Comparative Medicine Unit and the Veterinary Resources Branch, Division of Research Services

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SI

INSTITUTE AND LOCATION
NHLBI-IR/NIH/Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.90	0.15	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)

The purpose of this project is to compare the topographic patterns of increased endothelial surface permeability to the patterns of intimal lipid deposition of experimental atherosclerosis.

913

Project Description

Objectives: To compare the topographic patterns of increased endothelial surface permeability to the patterns of intimal lipid deposition of experimental atherosclerosis.

Methods Employed: A systematized necropsy procedure and a standardized format was developed for arranging and fastening the opened arterial tree to reflective panels for optical scanning and analysis. This standardized format makes possible quantitative analysis of the topographic distribution of intimal Evans blue dye accumulation (a measure of the permeability of the wall to plasma substances) and of intimal lipid deposition along the arterial tree of experimental animals. This standardized format may be expressed in a normalized coordinate system for automatic optical scanning and computer pattern analysis or may be used directly for comparative visual analysis. This format permits careful, detailed comparisons of areas of increased arterial wall permeability with areas of intimal lipid deposition at identical sites from animal to animal.

Major Findings: Application of these techniques to a recently completed study in experimental atherosclerosis in swine demonstrated that the disease pattern occurs with an extraordinary congruency from animal to animal. It was also shown that the patterns of increased endothelial permeability in normal animals are very similar to the patterns of intimal lipid deposition that occur in animals on an atherogenic regimen. At certain local sites, that were characterized by very high permeability in normal animals, a characteristic atheromatous lesion occurred which, under the above standardized necropsy procedures, appeared as a tri-colored lesion. These lesions were characterized by relatively normal surrounding intimal surface (white), a highly permeable (blue) center bordered by an annular but otherwise typical atheromatous plaque (red). With time, these lesions were shown to form mature atheromatous plaques in the abdominal aorta; however, in the thoracic aorta and subclavian artery these appear to mature more slowly. Some lesions in these areas appear to remain stationary for a matter of years maintaining their tri-color characteristics.

Significance to Biomedical Research and the Program of the Institute: The extraordinary congruency of the disease patterns from animal to animal in a given species, as well as to the patterns of permeability in the normal animal of that species represents a major finding of fundamental importance not only in linking the role of increased permeability to the subsequent development of this disease but also in permitting one to develop new strategies in experimental design to gain deeper insight into the associated atherogenic processes. The predictability of this process at given locations permits one to design studies to follow the sequence of events leading from the "earliest lesion" on through the complicated lethal atheromatous plaque. This capability was heretofore unavailable for lack of a standardized quantitative approach to the analyses of the disease topography and permeability topography. Moreover, the occurrence of the tri-color lesion provides the opportunity to study an apparent spectrum of atherogenic processes in a

very discrete anatomical region.

Proposed Course of the Project: Progress on this project was temporarily halted by loss of key personnel. New personnel have been trained and progress will resume. Analysis of the topographic data has involved relatively detailed computer data processing techniques and the necessity to change computer facilities has also hindered progress. Programs on the new system have now been developed which should allow statistical comparison between the maps of increased permeability and the occurrence of intimal lipid deposition. The standardized formating procedures have been incorporated into the routine necropsy procedures for all of our animal colonies; and, when the above analytic techniques become operational, this new approach to quantitative pathology will be used to assess the influence of a variety of dietary and other factors on the evolution of this important disease process. This methodology will make possible an approach to a number of fundamental questions: Why do some regions of moderate endothelial permeability progress only to the fatty streak lesions whereas others go on to mature atherosclerotic lesions? Why do certain local regions of extraordinarily high permeability appear to be relatively immune to lipid deposition? What is the sequence of events leading from a fatty streak to a lethal atheromatous plaque? How are the pattern of streaking and plaquing influenced by hemodynamic alterations, hematologic manipulations, dietary triglycerides, and exercise?

Publications

Fry, D.L. Hemodynamic forces in atherogenesis. Cerebrovascular Diseases. pp. 77-95, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02815 01 EA

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
A quantitative autoradiographic method for the measurement of radiolabeled protein distribution across arterial tissue

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

PI:	D.L. Fry	Chief, Lab. of Exptl. Atherosclerosis	EA NHLBI-IR
OTHER:	R.W. Mahley	Head, Comparative Atherosclerosis and Arterial Metabolism Section	EA NHLBI-IR
	S.J. Lewis	Histologist	EA NHLBI-IR
	F. Plowman	Mechanical Engineer	EA NHLBI-IR

COOPERATING UNITS (if any)
NONE

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SE

INSTITUTE AND LOCATION
NHLBI-IR/NIH/Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.20	0.20	1.00

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 quantitative autoradiographic technique was developed using electron probe microanalysis to determine the developed silver distribution across the autoradiograph. It was shown that the local tissue concentration of radiolabeled protein was linearily related to the silver signal from the electron probe system.

916

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The purpose of this research was to develop a quantitative autoradiographic technique for the measurement of radiolabeled protein distribution across the arterial wall.

Methods Employed: Excised canine aortas were placed in a specially designed device which permitted exposure of the intimal surface of the arterial tissue to radiolabeled albumin under controlled experimental conditions. Selected exposure periods and concentrations of radiolabeled albumin were chosen to vary the uptake of radiolabeled protein by the tissue at various sites along the vessel. In one set of experiments, uptakes were varied from zero to levels of activity in excess of those to be encountered in any experimental application of this technique. In the other set of experiments, paired wells were chosen such that adjacent sites would have the same tissue concentration of radioactivity and thus the distribution of activity at each site could be compared by independent techniques. By one technique, the radioactivity in 15 μm en face serial microtomy sections taken across the wall was measured directly. The other technique was quantitative autoradiography which was done on a matched tissue specimen. Following photographic development, the distribution of silver induced by the underlying radioactivity was quantified by electron probe microanalysis.

Major Findings: The volume averaged signal from the electron microprobe analysis of the silver was found to be directly proportional to the volume averaged tissue concentration of radiolabeled protein from zero to a range in excess of that expected in any application of this technique. Similarly, the comparison of the distribution of the silver across the autoradiograph was congruent with the distribution of radioactivity across the wall as determined by the serial microtomy sections.

Significance to Biomedical Research and the Program of the Institute: The above work establishes a new methodology by which it is possible to determine with very high resolution (within a micron or so) the concentration of radiolabeled proteins across fixed tissue that closely approximates the immediately prefixation distribution. Although this technique should have broad application to study of radiolabeled protein distributions across other tissues, its major significance in the present work is that it allows one to measure one of the principal variables in transvascular transport processes, i.e., the concentration gradients that drive the macromolecular flux. This technique will make possible the study of local transport processes in arterial tissue and their role in atherogenesis.

Proposed Course of the Project: A manuscript describing this methodology has been submitted to the American Journal of Physiology. This technology is being applied to studies of transvascular macromolecular transport in relation to atherogenesis.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02816 01 EA

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
The combined convective and diffusive transport of EBD albumin into the deendothelialized aortic wall

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

PI: D.L. Fry Chief, Lab. of Exptl. Atherosclerosis EA NHLBI-IR

COOPERATING UNITS (if any)
NONE

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SI

INSTITUTE AND LOCATION
NHLBI-IR/NIH/Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.90	0.15	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)

Macromolecular transport into the arterial wall occurs by diffusive as well as convective processes. The present studies, for the first time, quantified the relative order of magnitude of diffusive forces as compared to convective forces in transvascular transport across the deendothelialized aortic wall.

918

Project Description

Objectives: It was the purpose of these studies to measure the uptake of Evans blue dye (EBD) complexed albumin by the deendothelialized aortic wall that was related to diffusive forces and that was related to a superimposed positive as well as negative transmural pressure of 100 mm Hg pressure.

Methods Employed: Specially prepared, rapidly excised descending thoracic aortas from dogs were placed in a specially designed multichambered device in which the chemical milieu on the intimal surface as well as the transmural pressure could be under experimental control. Albumin was labeled with EBD and placed in the chambers on the intimal surface of the vessel. The well chambers along the aorta were divided into sets of three. One well in each set was maintained at zero transmural pressure, one well at a positive transmural pressure, and the third at a negative transmural pressure of 100 mm Hg pressure. Four sets of wells along the artery allowed four different exposure times to be studied such that three different curves could be constructed, each with four exposure time points on it representing the corresponding uptake of EBD albumin. One curve represented the uptake with 100 mm Hg positive pressure, one with 100 mm Hg negative pressure, and one with zero transmural pressure.

Major Findings: Comparison of the ordinate differences on these three curves allowed calculation of the increment of uptake induced by the pressure as compared to the uptake by purely diffusive forces. Comparison of the uptake curve from the positive transmural pressure to that for the negative transmural pressure allowed calculation of the increment increased influx into the wall related to the pressure alone and also to the imposed degree of stretch alone. The pressure driven flux into the wall was found (at physiologic levels of pressure of 100 mm Hg) to be of the same order as that for the steady state diffusive flux into the wall. Thus, in considering transport into the wall, both driving forces, diffusive and convective, must be taken into account; and, moreover, under conditions of hypertension, it is likely that the pressure driven or convective flux may become the predominant factor.

Significance to Biomedical Research and the Program of the Institute: A large body of evidence both from this laboratory as well as from others indicate that the atherogenic precursors probably come from the blood phase into the wall as various complexes of proteins. Moreover, the cholesterol rich remnants from metabolism of these lipoprotein substances are probably carried out of the wall by other protein or detergent-like substances which are also transported through the wall. While albumin itself can carry both fatty acids and some cholesterol, its transport is of major interest to us as a prototype model for macromolecular transport and binding in the intimal space. Thus, while of inherent interest in albumin and fatty acid transport, the above studies begin to place, for the first time, macromolecular transport in vascular tissue on a firm physical-chemical footing. With respect to atherogenesis, the implications of diffusive transport are quite

different from those of convective transport. As the relative importance of these transport mechanisms come into clearer perspective, it should be possible to gain deeper insights into a number of the puzzles presented by this disease process; why do fatty streaks occur at particular sites along the aorta; what is the reason for the curious propensity of certain sites to go on to develop the lethal atheromatous plaque; what causes the imbalance between the transport of atherogenic precursors into the wall and the transport of the cholesterol rich end products out of the wall?

Proposed Course of the Project: Data from the above studies is currently being processed and several physical, chemical models of transport are being tested. A manuscript is in preparation. These studies are being repeated with radiolabeled albumin and will be extended to muscular arteries.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02817 01 EA

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Protein modification and apoprotein characterization of plasma lipoproteins

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

PI:	R.W. Mahley	Head, Comparative Atherosclerosis and Arterial Metabolism Section	EA NHLBI-IR
	K.H. Weisgraber	Expert	EA NHLBI-IR
OTHER:	E. Gross	Head, Section on Molecular Structure	NICHHD

COOPERATING UNITS (if any)
Meloy Laboratories, Springfield, VA - NHLBI Contract No. NO1-HI-3-2926

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION
Comparative Atherosclerosis and Arterial Metabolism Section

INSTITUTE AND LOCATION
NHLBI-IR/NIH/Bethesda, MD 20014

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)

The purposes of this project are: 1) selective modification of amino acid residues of plasma lipoproteins and determination of the effect of such modifications on lipoprotein interaction with cultured cells, 2) isolation and characterization of the plasma lipoprotein apoproteins from man and lower species, and 3) establishment of apoprotein homology among various species.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: 1) To modify plasma lipoproteins with various protein reagents which react with specific amino acid residues, to study the effect of these modifications on the physical and chemical properties of the lipoprotein, and to determine whether these modifications alter cell surface receptor binding and/or degradation by fibroblasts and smooth muscle cells; 2) To isolate and characterize apoprotein from the plasma lipoproteins of man and of lower species and to establish homologies and differences of these apoproteins among the various species.

Methods Employed: Several chemical reagents, when allowed to react with proteins, are known to possess rather selective specificity for various amino acid residues. These reagents have been used successfully to modify numerous enzymes in an attempt to determine which amino acids are at or in the proximity of the "active site." We have carried out the reversible modification of the arginyl residue of the apolipoproteins of LDL and HDL_c using 1,2-cyclohexanedione. This reagent is specific for arginine under the conditions employed and is removed from arginine by treatment with hydroxylamine. The extent and kinetics of the arginine modification were determined by amino acid analysis. In addition, the effect of the modification on the binding, internalization, and degradation of the lipoproteins by cells in tissue culture was studied. A list of reagents which have been and will be employed and the respective amino acid residues with which they react includes: cyclohexanedione, arginine; N-acetylimidazole, lysine; trinitrobenzene sulfonate, lysine; B-mercaptoethanol, cysteine; N-ethylmaleimide, cysteine; iodoacetamide, cysteine; mercuribenzoate, cysteine; bromophenacyl bromide, histidine; and diamide, oxidation of sulfhydryl groups.

The plasma lipoprotein apoproteins were isolated by Sephadex and DEAE column chromatography and by preparative SDS gel electrophoresis. Characterization of the purified apoproteins included amino acid analysis, immunochemical properties, and molecular weight determination.

Major Findings: 1) We have found that selective modification of the arginyl residue of human LDL and canine LDL or HDL_c by cyclohexanedione abolishes the high-affinity binding of these lipoproteins to normal human fibroblasts. Amino acid analysis of the CHD-modified lipoproteins showed that approximately half of the arginyl residues, but no other amino acid residue, were involved. The physical and chemical properties of the lipoproteins, including lipid composition, were unchanged. Following removal of the CHD, the binding capacity of the lipoproteins was restored. These results demonstrated that the high-affinity binding specificity of LDL and HDL_c was localized to the apoprotein moiety and that arginine might be directly involved or in close proximity to the lipoprotein recognition site. 2) We have isolated an apoprotein with a molecular weight of ~48,000 from the d<1.006 fraction of patients with Type III hyperlipoproteinemia and from a subfraction of normal human d=1.063-1.125 (HDL_2) obtained by block electrophoresis. Treatment of this apoprotein with disulfide reducing agents resulted in the formation of two subunits (37,000 and 10,000 MW). The 37,000 MW component co-electrophoresed with the arginine-rich apoprotein (ARP), reacted with immunochemical identity, and had an amino acid composition indicative of the

ARP. We propose that this 48,000 MW apoprotein is a pro-apoprotein of the arginine-rich apoprotein (pro-ARP). In tissue culture, the subfraction of HDL_2 which contained pro-ARP and A-I as major apoproteins (lacking apo-B) was relatively incapable of displacing ^{125}I-LDL from cell surface receptors of fibroblasts and behaved like HDL_3 (d=1.125-1.21). However, following reduction with conversion of pro-ARP to ARP, this lipoprotein competitively displaced ^{125}I-LDL (50% displacement at 25 µg of protein) and was bound, internalized, and degraded similar to LDL. Reduction of LDL and HDL_3 did not decrease or enhance their binding activity. The pro-ARP may represent a functionally inactive form of the ARP and may modulate binding. 3) We have isolated the rat ARP from various rat lipoproteins (VLDL, HDL_1, HDL_c, and HDL) and established the identity of the ARP from these various lipoproteins, as well as homology with human swine, canine, and Patas monkey ARP by amino acid analysis, co-electrophoresis, and immunochemistry. The ARP was isolated by gel filtration, DEAE chromatography, and elution from SDS gels. Purified ARP samples exhibited heterogeneity in their elution profiles on DEAE chromatography and showed multiple bands of Tris-urea gel electrophoresis. These multiple DEAE peaks were shown to be identical by amino acid and immunochemical analysis. However, differences in the sialic acid contents of the peaks were detected. These sialic differences appear to be responsible in part for the ARP heterogeneity.

Significance to Biomedical Research and the Program of the Institute: Determination of the apoprotein content of various lipoproteins and correlation of apoprotein content with metabolic behavior is important to our understanding of the lipoproteins and their role in cardiovascular heart disease. The selective modification of amino acid residues of specific lipoproteins and the effects of such modification on the binding, internalization, and degradation of these lipoproteins provides insight into the factors which are involved in the metabolism of lipoproteins by cells in culture. Cellular metabolism may be an important regulatory mechanism of the in vivo levels of the various lipoproteins.

The specific protein constituents (apoproteins) of the various classes of plasma lipoproteins appear to regulate the metabolism of the lipoproteins and to determine the fate of each class. Identification of these constituent proteins and their characterization are essential to the understanding of lipoprotein metabolism. The importance of specific apoproteins (B and arginine-rich apoprotein, in particular) in the regulation of cellular cholesterol metabolism in fibroblasts and arterial smooth muscle cells has been established. Identification of the precise physicochemical properties responsible for the interaction of certain lipoproteins, and not others, with cell surface receptors may provide insight into the regulatory mechanism of the deposition of cholesterol in the arterial wall.

Proposed Course of the Project: The project will continue along the lines indicated above.

Publications

Mahley, R.W., T.L. Innerarity, R.E. Pitas, K.H. Weisgraber, J.H. Brown, and E. Gross. Inhibition of lipoprotein binding to cell surface receptors of fibroblasts following selective modification of arginyl residues in the arginine-rich and B-apoproteins. J. Biol. Chem. In press.

Weisgraber, K.H., R.W. Mahley, and G. Assmann. The rat arginine-rich apoprotein and its redistribution following injection of iodinated lipoproteins into normal and hypercholesterolemic rats. Atherosclerosis. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02818 01 EA

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

The role in hyperlipoproteinemia of a high-density lipoprotein induced by cholesterol feeding

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

PI:	T.P. Bersot	Clinical Associate	EA NHLBI-IR
OTHER:	R.W. Mahley	Head, Comparative Atherosclerosis and Arterial Metabolism Section	EA NHLBI-IR
	D.L. Fry	Chief, Laboratory of Experimental Atherosclerosis	EA NHLBI-IR

COOPERATING UNITS (if any)
MDB Clinical Service

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION
Comparative Atherosclerosis and Arterial Metabolism Section

INSTITUTE AND LOCATION
NHLBI-IR/NIH/Bethesda, MD 20014

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)

Outpatient normal volunteers ate 6 eggs daily and diets high in saturated fat. The plasma cholesterol concentration increased in five subjects during the first two weeks but had returned to pre-study levels when the study was terminated at the end of four weeks. In the high-density lipoproteins of all subjects, a subfraction changed in that it developed an increased content of the arginine-rich apoprotein (ARP). When the ARP content increased, the HDL was able to bind to cells in tissue culture and inhibit cellular cholesterol synthesis. HDL without ARP were not able to bind to high affinity binding sites.

925

Project Description

Objective: 1) To study the effects of eating cholesterol upon plasma lipoprotein metabolism. 2) To determine if eating cholesterol has any effect upon the accumulation of cholesterol in cells.

Methods Employed: 1) Normal volunteers were fed 6 eggs daily for four weeks. 2) Plasma cholesterol and triglyceride concentrations were measured and lipoprotein subfractions were isolated by previously described methods. 3) Compositions of the isolated lipoprotein fractions were determined by standard techniques. 4) Isolated lipoprotein fractions were tested in cell culture experiments for their ability to bind to fibroblasts and influence cellular sterol metabolism.

Major Findings: Cholesterol-feeding six normal subjects had no effect upon plasma cholesterol concentrations after four weeks. The high density lipoproteins changed in that a subfraction of HDL_2 developed an increased content of the arginine-rich apoprotein and this fraction then became able to transfer cholesterol into cells in vitro.

Significance to Biomedical Research and the Program of the Institute: The major significance of these studies is that eating cholesterol induces the production of an HDL which can cause cholesterol to be transferred to cells. Thus, eating cholesterol may not increase plasma cholesterol concentrations but may still induce the deposition of cholesterol in tissues.

Proposed Course of the Project: Further investigation is now required to separate the effects of saturated fat and cholesterol feeding upon plasma cholesterol concentrations and the plasma lipoproteins. Such studies will require inpatients so that careful control of dietary fat content can be maintained. Differing responses of plasma cholesterol concentrations among various subjects fed cholesterol also requires exploration.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02819 01 EA

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)
Plasma exchange in homozygous familial hypercholesterolemics

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

PI:	T.P. Bersot	Clinical Associate	EA NHLBI-IR
OTHER:	H.B. Brewer	Chief, Molecular Disease Branch	MD NHLBI-IR
	E. Schaefer	Staff Associate	MD NHLBI-IR
	R.I. Levy	Director	NHLBI
	A.B. Deisseroth	Head, Experimental Hematology Section	C PO

COOPERATING UNITS (if any)
MDB Clinical Service
Pediatric Oncology Branch, NCI

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION
Comparative Atherosclerosis and Arterial Metabolism Section

INSTITUTE AND LOCATION
NHLBI-IR/NIH/Bethesda, MD 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.75	0.25	0.5

CHECK APPROPRIATE BOX(ES)

[X] (a) HUMAN SUBJECTS [ ] (b) HUMAN TISSUES [ ] (c) NEITHER

[ ] (a1) MINORS [ ] (a2) INTERVIEWS

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

Three patients with homozygous familial hypercholesterolemia have been treated by monthly plasma exchange to lower plasma cholesterol concentrations. One patient treated for 17 months has had marked diminution of cutaneous xanthomas as a result of his exchanges. Two others exchanged for shorter periods of time have had no objective improvement. None of the patients has had any change symptomatically.

927

Project Description

Objective: 1) To assess the efficiency of chronic plasma exchange as a therapeutic tool to lower plasma cholesterol concentrations and reduce the tissue burden of cholesterol in HFH patients. 2) To study the effects of plasma exchange upon plasma lipoproteins.

Methods Employed: Plasma exchanges are performed using a continuous flow centrifuge into which the anticoagulated patient's blood flows. The patient's plasma is collected and replaced with a plasma protein fraction containing no lipoprotein cholesterol.

Major Findings: The regression of xanthomas in the patient treated for 17 months is the first demonstration that this procedure can reduce tissue cholesterol burdens. There has been one significant side effect, and this was a single episode of ischemia occurring during one exchange.

Significance to Biomedical Research and the Program of the Institute: Patients with HFH are resistant to drugs used to lower plasma cholesterol at present. They have a poor prognosis as a result of this. Plasma exchange offers an opportunity to reduce tissue cholesterol burdens and to determine if vascular atheromata will regress.

Proposed Course of the Project: The project will continue, for therapeutic purposes, until patients have undergone therapy for two years. At that point, repeat catheterization to perform coronary arteriography will be performed. If there is significant vascular improvement, the project will be continued indefinitely until more efficient and efficacious therapeutic means are available.

Publications

None

Annual Report of the Pathology Branch

Division of Intramural Research

National Heart, Lung, and Blood Institute

July 1, 1976 through September 30, 1977

This branch is concerned primarily with the study of structural alterations produced by various cardiovascular and pulmonary diseases. Structural alterations are studied at gross, light microscopic, ultrastructural and histochemical levels. Studies during this period focused on coronary, valvular, myocardial, and congenital cardiac diseases.

CORONARY HEART DISEASE

A continuing major undertaking of this laboratory is the examination in more or less serial fashion of the major extramural coronary arteries in a number of different conditions. In the past a single question has been asked regarding the status of each major coronary artery in a particular condition, i.e., is the artery >75% narrowed in cross-sectional area or not and if not what is the maximal degree of luminal narrowing. Studies during the above period have shifted from a qualitative examination of the major extramural coronary arteries to a quantitative examination such that it's possible to determine how many centimeters of a particular coronary artery are narrowed >75%, the number narrowed between 51 and 75%, the number narrowed between 25 and 50% and the number narrowed <25%. In the past it's not been possible to show any differences in the degrees of luminal narrowing in patients with pure angina pectoris, sudden coronary death and in patients with fatal acute myocardial infarction. It is hoped that the more quantitative analysis of the major coronary arteries will allow some answer to the question as to why one patient with severe coronary heart disease dies suddenly, another dies of acute myocardial infarction and another dies of progressive congestive failure after healing of a transmural myocardial infarction. These studies are now in progress in regard to each of these three conditions.

a) Status of the coronary arteries in the nephrotic syndrome. This was the first study which quantitated the amount of coronary luminal narrowing precisely in each of the 4 major coronary arteries. In this study the major extramural coronary arteries in 20 patients (avg 24 years) with nephrotic syndrome (from systemic lupus erythematosus or diabetes mellitus or chronic idiopathic glomerulonephritis) was determined and the observations compared to those in 14 control subjects (avg 29 years). The 20 patients with nephrotic syndrome had significantly more coronary luminal narrowing by atherosclerotic plaques than did the control subjects. The lumens of 1 or more of the 4 major coronary arteries were narrowed >75% in cross-sectional area in eight of the 20 nephrotic patients and in one of the 14 control subjects. This difference in degree of coronary disease was more striking when the percent of narrowing in the entire coronary tree was examined: of the 290 cm of coronary artery examined in the 20 nephrotic patients, the lumen in 88 cm (30%) was >50% narrowed in cross-sectional area whereas 288 cm of coronary artery examined in the 14 control subjects the lumen in only 5.5 cm (2%) was narrowed to this degree. This study did not determine the cause of the ac-

celerated atherosclerosis in the nephrotic patients but certainly hypercholesterolemia, systemic hypertension, corticosteroid therpay and increased clotting tendencies in these patients probably all contributed to this acceleration.

b) The extramural and intramural coronary arteries in juvenile diabetes mellitus. This study was also a quantitative analysis of the degrees of coronary arterial narrowing in nine patients with juvenile diabetes. The observations in them were compared to those in nine control subjects of simialr average age (29 years). The juvenile diabetic patients had significantly more extramural coronary luminal narrowing by atherosclerotic plaques than did the control subjects. Lumens of one or more of the 4 major extramural coronary arteries were narrowed >75% in cross-sectional area in 6 of the diabetic patinets and in none of the 9 control subjects. This difference in degree of narrowing of the epicardial coronary arteries was even more striking when the percent of narrowing of entire lengths of the 4 major coronary arteries were examined: of the 191 cm of major coronary artery examined in the nine diabetic patients the lumen in 90.5 cm (47%) was >50% narrowed in cross-sectional area whereas of 155 cm of coronary artery examined in the nine control subjects the lumen of only 2 cm (1%) was narrowed to this degree. Only minor degrees of intimal fibrous proliferation was noted in the intramural coronary arteries in the diabetic patients.

c) Coronary anastomotic sites of aortocoronary bypass grafts. This study examined the status of the native coronary arteries at necropsy in the vicinity of the coronary anastomosis of saphenous vein aortocoronary bypass grafts in 20 patients with severe coronary heart disease. The interesting finding was that the lumina of 44% of the coronary arteries within the first 2 cm distal to the anastomosis were >75% narrowed in cross-sectional area by atherosclerotic plaques and the native coronary artery at the site of the anastomosis was >50% narrowed in nearly half of the patients. This study clearly showed that the coronary anastomosis should be placed more distal to where they were placed at least in this group of patients.

d) Changes in saphenous veins used as aorto-coronary bypass grafts. A continuing study in this laboratory in the past 2 years has been the examination histologically of the saphenous veins used as aorto-coronary bypass conduits. This study showed that there were 3 major changes in these veins used as conduits. They were - medial fibrous replacement, adventitial fibrous proliferation, and intimal proliferation. The probable mechanisms of each of these 3 changes was predicted on the basis of these morphologic studies. It is clear that all saphneous veins used as bypass conduits undergo change.

e) Medial calcinosis of Monckeberg. A 41-year-old woman was studied at necropsy who had evidence of massive medial calcification of the intra-abdominal arteries as well as medial calcification of the peripheral arteries of the arms and legs. Thorough examination of the coronary arteries in the method described eariler also disclosed calcification of the media of these coronary arteries. Documentation of medial calcinosis in the coronary arteries had never been described previously and furthermore, documentation of medial calcinosis involving the abdominal arteries has been extremely rare. This type of arterial disease appears to be far more common in diabetic patients than in non-diabetic patients.

VALVULAR HEART DISEASE

A continuing activity of this branch over the last several years has

been the study of patients with valvular heart disease to gain more information regarding the natural history of the various valvular lesions and the complications of prosthetic cardiac valves.

a) Aschoff bodies in operatively excised atrial appendages and in papillary muscles. A major study this past year was the analysis of nearly 500 patients undergoing various mitral-valve operations to seek the frequency of Aschoff bodies in left atrial appendages and in excised papillary muscles. Aschoff bodies in the heart in patients undergoing cardiac operations is dropping (in this study only 21%) and that Aschoff bodies in virtually 99% of patients indicate the presence of mitral stenosis.

b) Aschoff bodies at necropsy in valvular heart disease. As a sequence to the above study, histologic sections of heart were examined in 543 necropsy patients 14 years of age with severe fatal chronic valvular heart disease. Aschoff bodies were found in only 11 patients (2%). This is a far lower percentage than has ever been observed previously and possibly indicates that rheumatic fever is either being better treated or controlled or is less virulent than it possibly was in the pre-antibiotic era.

c) "Mitral Stenosis" secondary to "Massive" mitral annular calcific deposits. It has been predicted for about two decades that if calcific deposits in the mitral annulus become heavy enough that they can produce mitral obstruction. This observation however, has never been confirmed hemodynamically but it was confirmed in 4 patients described in a study from this laboratory who had the documentation by hemodynamic studies and confirmation at necropsy that the calcific deposits were limited to the mitral annular region.

d) Healed left-sided infective endocarditis. A continuing study in this branch thru the years has been analysis of patients with active infective endocarditis. Nearly 150 patients with fatal active infective endocarditis have been studied in detail at necropsy. Studies during this past year concerned patients with active infective endocarditis which had been healed by antibiotic therapy and the patients either died subsequently or had to undergo valve replacement because of damage to the valve from the period of activity. Interestingly, there have been virtually no previous reports on morphologic changes in patients with healed infective endocarditis. Analysis of all the patients with fatal infective endocarditis in this laboratory has shown that this continues to be a devastating disease and that of the patients cured of the infection by antibiotics only 1/3 are left without residual valve damage.

e) Structural changes in porcine xenographs used as substitute cardiac valves. A continuing study in this laboratory has been evaluation of the various prosthetic cardiac substitute valves. The porcine xenograph has received little attention from the morphologic aspect. Consequently 51 Hancock glutaraldehyde-preserved porcine heterograft bioprostheses from 41 patients was studied. The conclusions from this study are that all of these tissue valves undergo changes, that at the time periods studied, i.e., up to 74 months, that gross changes are infrequent but that histologic changes and ultrastructural changes are quite significant. These morphologic studies suggest that the porcine valve prosthesis will break down but exactly what the time period of collapse so to speak will be is unclear at this time.

f) Aortic dissection after aortic replacement. Two patients were described who had dissection of aorta following aortic valve replecement. This rare complication is discussed in view of other investigators observations of

this association.

MYOCARDIAL DISEASE

a) Sarcoidosis of the heart. A major study of this unit during the above time period was analysis of 35 necropsy patients with cardiac sarcoidosis. Analysis of these patients disclosed that most of those with caridac sarcoidosis granulomas in their hearts died from cardiac disease, namely arrhythmia, progressive heart failure, recurring pericardial effusion, tachy arrhythmias, particularly ventricular tachycardia, and complete heart block. Although corticosteroid therapy tends to cause fibrous replecement of myocardial sarcoid granulomas, a possible consequence of this medication is the development of ventricular aneursym. It was interesting to learn that most of the patients with cardiac sarcoidosis at least causing cardiac dysfunction presented initially with manifestations related to the heart, and that most patients with cardiac sarcoidosis had little or no clinical evidence of dysfunction of an organ system other than the heart. This study also clearly indicated that patients previously described as having idiopathic granulomatous myocarditis probably had cardiac sarcoidosis.

b) Trichinosis causing extensive ventricular mural endocarditis. The mural endocardium of the heart for some reason appears to react very unfavorably to high blood eosinophilia. A patient is described who developed severe destruction of mural endocardium of both right and left ventricles associated with severe blood eosinophilia secondary to acute trichinosis.

c) Cardiomyopathy and myocarditis. The necropsy cases sectioned in this branch through the years under primary cardiomyopathy were examined and compared to those cases diagnosed as acute myocarditis. The striking observation was that acute myocarditis today is extremely uncommon and is a rare cause of death whereas most patients dying of heart muscle disease have no inflammatory cells whatsoever in their myocardium at necropsy.

CONGENITAL HEART DISEASE

a) Aortic valve atresia assocaited with ventricular septal defect. Last year a group of 73 necropsy patients with aortic valve atresia were described and 4 were observed to have associated ventricular septal defect. This report analyzed those 4 patients with ventricular septal defect in detail and showed that it was possible to separate patients clincally with aortic valve atresia from those without ventricular septal defect. This differentiation is important because patients without ventricular septal defect are not operative candidates whereas those with this defect, potentially are.

MISCELLANOUS

a) Canine hearts after 24-hour preservation and orthotopic transplantation. This study summarized gross and histologic observations in canine hearts studied after 24-hours of preservation and orthotopic transplantation. These results indicated that oxygenated perfusion with a hypothermic, hypertonic intracellular solution may permit improved transplant survival after extended cardiac preservation.

b) Fibrosing mediastinitis causing pulmonary arterial hypertension. Two patients were described with severe pulmonary arterial hypertension without pulmonary venous hypertension resulting from fibrosing mediastinitis. The latter condition rarely is a cause of pulmonary arterial obstruction but this study clearly shows that it may cause arterial obstruction even without pulm-

onary venous obstruction.

c) Primary sarcoma of the pulmonary trunk and/or right or left main pulmonary trunk. Two patients with this rare sarcoma were described and its differentiation from a number of conditions causing right ventricular hypertension were discussed.

d) Examining the heart at necropsy. Through the years there have been a number of methods described to examine the heart at necropsy. This piece described the manner in which hearts are examined at necropsy in this branch. Three principles were established and they are: 1) that the heart is fixed in formalin prior to its opening; 2) the heart is x-rayed after fixation; and 3) that the method chosen to incise the heart is determined by the type of cardiac disease present or suspected. These three principles have proved to be highly beneficial in examining hearts and although they may appear relatively self-evident they are rarely done in other units.

ULTRASTRUCTURAL SECTION

CARDIAC HYPERTROPHY

Previous studies from this unit and from other investigators have established that the ultrastructural features of cardiac hypertrophy vary according to the cause and the stage of the hypertrophy, and that different organelles of cardiac muscle cells have different responses to the stimulus of hypertrophy. As part of our continuing, long-range study of cardiac hypertrophy, this year we conducted studies of: the occurrence of spherical microparticles in myocardium, the features of left atrial hypertrophy in patients with mitral valvular disease, and the presence of myocardial degeneration as a function of age in patients with congenital heart diseases. In addition, reviews were undertaken of: 1) structural features of myocardial hypertrophy in humans, and 2) structural aspects of the contractile proteins in myocardium.

a) Spherical microparticles: Clusters of spherical microparticles that averaged 500 Å in diameter and were composed of dense cores surrounded by single trilaminar membranes were found in operatively obtained myocardial biopsis from 29 to 70 patients with various types of heart disease. These particles were consistently associated with interstitial fibrosis and with degeneration of the muscle cells. The spherical microparticles occurred along the outer surfaces on the sides and free ends of the muscle cells in areas of fibrosis, in the widened spaces between the membranes of partially dissociated intercellular junctions, and within cytoplasmic vesicles considered to be phagocytic. Spherical microparticles often were joined together by minute nexuses similar to those forming parts of intercellular junctions of muscle cells. Evidence was found, in the form of budding, to support the concept that spherical microparticles are derived from the plasma membranes as part of a process that mediates the remodeling of cellular surfaces, especially those of intercellular junctions undergoing dissociation.

b) Left atrial ultrastructure in mitral valvular disease. Light and electron microscopic observations were made on left atrial biopsies of 14 patients undergoing operative repair of mitral valvular disease. In fibrotic areas, present in all biopsies, the muscle cells were frequently isolated from each other and exhibited degenerative changes of variable severity. These changes consisted of: lysis of myofibrils, with preferential loss of myosin filaments; proliferation of Z-band material; increased content of cytoskeletal (100 Å diameter) filaments; proliferation of elements of sarcoplasmic reticu-

lum (SR), with formation of aggregates of hexagonally arranged tubules of free SR and of large complexes of cisterns of extended junctional SR; dissociation of intercellular junctions, with formation of spherical microparticles and intracytoplasmic junctions; and accumulation of lysosomal degradation products. These changes were considered to present the end stages of hypertrophy of atrial muscle cells.

c) Myocardial degeneration of congenital heart disease. A comparison was made of electron microscopic findings in operatively resected crista supraventricularis muscle from 11 patients aged 1 to 5 years (Group 1) and from 8 patients aged 30 to 53 (Group 2) with congenital heart disease associated with muscular obstruction to right ventricular outflow. Changes of hypertrophy (cells over 20 μ in diameter, lobulated nuclei, multiple intercalated discs, dilated T tubules and increased numbers of ribosomes) were present in both groups but were more prominent in the older patients. Marked interstitial fibrosis, cellular atrophy, myofibrillar disorganization, myelin figures, myofibrillar lysis, proliferation of smooth endoplasmic reticulum, lipid deposition, spherical microparticles associated with the plasma membranes of the muscle cells, intracytoplasmic junctions and thickening of the basal laminae of the muscle cells were entirely absent in the younger patients, although common in the patients over 30 years old. Other changes common in Group 2 (cellular disorganization, intramitochondrial glycogen and abnormal Z bands) were infrequent in Group 1. Ultrastrucutral changes of severe degeneration in the patients over 30 years old appeared to reflect the stresses of prolonged right ventricular hypertrophy and hypoxia and correlated with clinical cardiac dysfunction.

ENDOCARDIAL FIBROELASTOSIS

Histological and ultrastructural studies of left ventricular endocardium from 10 patients with endocardial fibroelastosis (EFE) revealed that the average size of the elastic fibers in thickened endocardium was much larger in the 4 patients with congenital EFE than in the 6 patients with acquired EFE (secondary to ischemic heart disease in 2 patients, to prosthetic cardiac valves in 3, and to irradiation to the chest in 1). Both components of normal elastic tissue (central, amorphous cores and peripheral microfibrils) were present in endocardial elastic fibers from each patient.

MYOCARDIAL DISEASES

a) Seleniun-vitamin E deficiency. Studies of the ultrastructure of cardiac muscle and blood vessels of pigs with experimentally induced selenium-vitamin E deficiency (a disease that occurs spontaneously in sheep, cattle, pigs, and poultry and is of considerable economic importance to farmers) were made in collaboration with Dr. John F. Van Vleet and his associates at Purdue University. The heart in this disease is characterized grossly by hydropericardium and by scattered pale streaks and patches of necrosis in the myocardium, especially the left ventricle. The damaged fibers have hypercontraction bands, myofibrillar lysis, and swelling, disruption and mineralizaition of mitochondria. These fiber lesions are accompanied by myocardial arteriolar damage consisting of segmental fibrinoid accumulation in vascular walls and by scattered fibrin thrombi. Both the cardiac muscle cell and the vascular lesions are regarded as resulting from liperoxidative damage to membranes that lack protection by the antioxidant system constituted by vitamin E and the selenoenzyme glutathione peroxidase.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03015-04 PA

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Endocardial Structure in Carcinoid Heart Disease

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 IR PA NHLBI
Other: William C. Roberts, Chief, Pathology Branch IR PA NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Pathology Branch

SE

Ultrastructure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.05	0.05

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 endocardial lesions in carcinoid heart disease are characterized by the presence of smooth muscle cells, myofibroblasts and fibroblasts embedded in a stroma that is rich in collagen, microfibrils and basement membrane material, but lacks elastic fibers.

935

PHS-6040
(Rev. 10-76)

Project Description: The carcinoid syndrome is associated with pathognomonic cardiac lesions which consist of plaque-like fibrous thickenings in mural and valvular endocardium. Ultrastructural study of these plaques in right atrium (2 patients) and tricuspid and pulmonic valve (1 patient) showed similar features. Most cellular elements were mature smooth muscle cells which varied from fusiform to stellate in shape and had greatly thickened, reduplicated basement membranes. Extracellular components consisted of: layers of normal-appearing collagen fibrils oriented parallel to the surfaces of the plaques and arranged in a cross-weaving pattern; 100 to 200 Å diameter microfibrils; layers of fibrillar material similar to basement membranes of smooth muscle cells, and dense spicules, 150 Å diameter and up to 800 Å in length. No elastic fibers or fibrin deposits were found. These findings suggest that carcinoid plaques result from stimulation of endocardial smooth muscle cells to produce collagen and basement membrane-like material; such stimulation may be intermittent, as evidenced by the layered arrangement of the plaques.

Publications: Ferrans, V.J., and Roberts, W.C.: The carcinoid endocardial plaque. An ultrastructural study. Hum Pathol 7: 387-409, 1976

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03018-03 PA

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Ultrastructural Features of Endocardial Fibroelastosis

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	IR PA NHLBI
Other:	Michael C. Fishbein, Guest Worker, Pathology Branch	IR PA NHLBI
	William C. Roberts, Chief, Pathology Branch	IR PA NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Pathology Branch

SL
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.30	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)

Histological and ultrastructural studies revealed that the average size of elastic fibers in thickened endocardium was much larger in congenital than in acquired endocardial fibroelastosis.

937

Project Description: Histological and ultrastructural studies of left ventricular endocardium from 10 patients with endocardial fibroelastosis (EFE) revealed that the average size of the elastic fibers in thickened endocardium was much larger in the 4 patients with congenital EFE than in the 6 patients with acquired EFE (secondary to ischemic heart disease in 2 patients, to prosthetic cardiac valves in 3, and to irradiation to the chest in 1). Both components of normal elastic tissue (central, amorphous cores and peripheral microfibrils) were present in endocardial elastic fibers from each patient. Ultrastructural identification of elastic fibers was facilitated by staining with silver tetraphenylporphin sulfonate.

Publications: Fishbein, M.C., Ferrans, V.J., and Roberts, W.C.: Histologic and ultrastructural features of primary and secondary endocardial fibroelastosis. Arch Pathol Lab Med 101: 49-54, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03023-03 PA

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Cardiac Structure in 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 IR PA NHLBI
Other: None

COOPERATING UNITS (if any)

None

LAB/BRANCH
Pathology Branch

S
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.05	0.05

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)

A detailed review of qualitative and quantitative ultrastructural changes in cardiac hypertrophy.

939

PHS-6040
(Rev. 10-76)

Projection Description: This project consisted of a detailed review of cardiac structure in hypertrophy, with emphasis on ultrastructural alterations of myocardium in the three stages of hypertrophy (developing hypertrophy, stable hyperfunction and cellular exhaustion) and on quantitative data derived from stereological analysis of electron micrographs from animal models of hypertrophy.

Publications: Ferrans, V.J.: Structural aspects of cardiac hypertrophy. To be published as a book chapter in Cardiac Hypertrophy, Morkin, E. (Ed.), New York, John Wiley & Sons

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03050-02 PA

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Skeletal Muscle Ultrastructure in Selenium-Vitamin E Deficiency in Chicks

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 IR PA NHLBI
Other: John F. Van Vleet, Purdue University School of Veterinary Medicine

COOPERATING UNITS (if any)

Purdue University School of Veterinary Medicine, Lafayette, Indiana

LAB/BRANCH
Pathology Branch

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Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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)

This study describes the histologic and electron microscopic features of the skeletal muscle degeneration and exudative diathesis that develop in chicks deficient in selenium and vitamin E.

941

PHS-6040
(Rev. 10-76)

Project Description: Chicks fed a semisynthetic basal diet deficient in selenium and vitamin E for 14 to 22 days developed skeletal myodegeneration and exudative diathesis. Chicks fed the basal diet supplemented with either 0.2 ppm selenium as selenite or 100 I.U. α tocopherol acetate/kg were protected from deficiency disease, but chicks fed the basal diet plus 0.4% L-cystine were not protected. Pectoral muscles of deficient chicks were red and edematous. Light and electron microscopic study of affected muscles revealed fibers with hyaline and granular degeneration. In hyalinized fibers, the initial ultrastructural alterations were increased density of the sarcoplasm and myofibrils, dilatation of sarcoplasmic reticulum, formation of subsarcolemmal vacuoles, and disruption of mitochondrial membranes. In later stages, alterations in these fibers included myofibrillar disruption and lysis, nuclear pyknosis and lysis, disruption of the plasma membrane with persistence of basal lamina and scattered adhering satellite cells, and eventual invasion by macrophages. In fibers with granular degeneration, the ultrastructural findings included decreased density of the sarcoplasm, prominent mitochondrial swelling and distortion, and multiple foci of myofibrillar lysis that eventually coalesced to produce generalized lysis. Prominent vascular lesions associated with exudative diathesis were present in the degenerated muscle but were not considered to precede the development of the fiber alterations. Affected vessels had endothelial cells with mitochondrial damage and accumulations of cytoplasmic dense bodies, and areas of endothelial disruption with adhering fibrin thrombi. The results of these studies support the concept that selenium and vitamin E play a role in protecting cellular membranes from lipoperoxidation.

Publications: Van Vleet, J.F., and Ferrans, V.J.: Ultrastructural changes in skeletal muscle of selenium-vitamin E deficient chicks. Am J Vet Res 37: 1081-1089, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03051-02 PA

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Morphology of Cardiac Rhabdomyomas

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 IR PA NHLBI

Other: John J. Fenoglio, Division of Cardiovascular Pathology, AFIP
Washington, D. C.

Hugh A. McAllister, Division of Cardiovascular Pathology, AFIP
Washington, D. C.

COOPERATING UNITS (if any)

Division of Cardiovascular Pathology, Armed Forces Institute of Pathology, Washington, D. C.

LAB/BRANCH

Pathology Branch

SE

Ultrastructure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.30	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)

Clinical and pathologic features of cardiac rhabdomyomas in 36 patients are described, including 7 patients in whom the tumors were examined by electron microscopy. It is concluded from these studies that rhabdomyomas of the heart are definitely derived from cardiac muscle cells and probably represent hamartomas rather than true tumors.

943

Project Description: Clinical and pathologic features of cardiac rhabdomyomas from 36 patients are described. Of these tumors, 90% were multiple and 78% occurred in children under one year of age. In 56% of the patients at least one of the tumor masses was intracavitary and in 42% it obstructed one or more of the cardiac valves. The tumors occurred with equal frequency in the right and left ventricles and in subepicardial and subendocardial locations. Ultrastructural study of tumors from 7 patients revealed similar features: paucity of myofibrils, which were arranged either parallel to the cell surfaces or in strands radiating toward the center; randomly located intercellular junctions, which had specialized components similar to those of intercalated discs of normal myocardium; and numerous leptofibrils, which were arranged either peripherally or in centrally located, spiralling clusters. It is concluded that cardiac rhabdomyomas are derived from muscle cells and probably represent hamartomas rather than true tumors.

Publications:

Fenoglio, Jr., J.J., McAllister, Jr., H.A., and Ferrans, V.J.: Cardiac rhabdomyoma: a clinicopathologic and electron microscopic study. Am J Cardiol 38: 241-251, 1976.

Fenoglio, Jr., J.J., Diana, D. J., Bowen, T.E., McAllister, H.A., Jr., and Ferrans, V.J.: Ultrastructure of a cardiac rhabdomyoma. Hum Pathol (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03052-02 PA

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Skeletal Muscle Ultrastructure in Selenium-Vitamin E Deficiency in Pigs

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 IR PA NHLBI
Other: John F. Van Vleet, Purdue University School of Veterinary Medicine
George R. Ruth, Purdue University School of Veterinary Medicine

COOPERATING UNITS (if any)
Purdue University School of Veterinary Medicine, Lafayette, Indiana

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.30	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)

This study describes histologic and ultrastructural studies of skeletal muscle lesions present in pigs fed a semisynthetic diet deficient in selenium and vitamin E for 13 to 59 days. Major alterations observed consisted of hyaline degeneration of the muscle cells, with subsequent macrophagic invasion and muscle cell regeneration by myoblastic proliferation, fusion and differentiation into fibers with mature myofibrils.

945

Project Description: Light and electron microscopic studies were made of the lesions which developed in the skeletal muscles of 24 of 38 young growing pigs fed a semisynthetic diet deficient in selenium and vitamin E for 13 to 59 days. The major alterations in injured fibers progressed from hyaline degeneration, with subsequent macrophagic invasion and phagocytosis of disrupted sarcoplasm, to muscle fiber regeneration by myoblastic proliferation, fusion and differentiation into fibers with mature myofibrils.

The earliest electron microscopic alterations observed were myofibrillar lysis and disruption, with thick filaments persisting longest. Disruption of mitochondria, sarcoplasmic reticulum and plasma membranes was seen in fibers with myofibrillar alterations. The basal lamina of the sarcolemma remained after destruction of the enclosed sarcoplasm and served as a scaffold for subsequent regeneration. Stages of regeneration that were observed included myoblastic proliferation, fusion into cords and myotubes, and, finally, fibrillogenesis to restore contractile material.

The sequence of structural alterations found in this study was discussed in terms of current theories of the biochemical role of selenium and vitamin E in maintaining cellular integrity.

Publications: Van Vleet, J.F., Ruth, G. R., and Ferrans, V.J.: Ultrastructural alterations in skeletal muscle of pigs with selenium-vitamin E deficiency. Am J Vet Res 37: 911-922, 1976

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03053-02 PA

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
The Heart in the Hurler Syndrome

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 IR PA NHLBI
Other: Vicente G. Renteria, Guest Worker, Ultrastructure Sec IR PA NHLBI
William C. Roberts, Chief, Pathology Branch IR PA NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Pathology Branch

SL
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.30	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)

Cardiovascular lesions in 5 patients with the Hurler syndrome involved the coronary arteries, the 4 cardiac valves, the mural endocardium, the myocardium and the aorta. These sites contained large, clear cells with deposits of acid mucopolysaccharides; in addition, cardiac muscle cells and smooth muscle cells contained deposits of glycolipid material. These changes led to valvular deformities and to extremely severe narrowing of the coronary arteries.

947

Project Description: Clinical and morphologic features of the cardiovascular system are described in five necropsy patients with the Hurler syndrome. In all five patients the coronary arteries, four cardiac valves, mural endocardium of all four chambers, myocardial walls and aorta were affected in a characteristic manner. All of these sites contained large clear cells known as Hurler cells (readily visible by light microscopy). In addition, granular cells were observed in semithin (1 micron) sections and by electron microscopy in the coronary arteries, atrioventricular valves and in myocardial interstitium. These latter cells appear to produce collagen in an abnormal way and are probably responsible for the heavy deposits of collagen in the cardiovascular system of patients with the Hurler syndrome. In the cardiac muscle cells, smooth muscle cells of the coronary arteries and in fibroblasts, wherever located, deposits of acid mucopolysaccharides (AMP) and glycolipids usually also were observed. The AMP deposits were observed easily by light microscopy except in the cardiac muscle cells and in them they were seen only by electron microscopy. The glycolipid deposits, observed only by examination of 1 micron thick sections or by electron microscopy, have not been observed previously in coronary arteries or in myocardial cells.

The infiltration into the heart by the above cells and deposits in all 5 patients resulted in severe narrowing of the extramural coronary arteries, considerable thickening of the cardiac valves (the left-sided ones more than the right-sided ones), generalized thickening of mural endocardium, and "stiffening" of the myocardial walls. Thus, the cardiovascular lesions in the Hurler syndrome are specific and life-threatening.

Publications: Rentería, V.G., Ferrans, V.J., and Roberts, W.C.: The heart in the Hurler syndrome. Gross, histologic and ultrastructural observations in five necropsy cases. Am J Cardiol 38: 487-501, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03060-01 PA

PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Status of the Coronary Arteries in the Nephrotic Syndrome

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

PI:	R. Charles Curry, Jr., M.D.	Guest Worker	PA	NHLBI
Other:	William C. Roberts, M.D.	Chief	PA	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	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)

This study describes the status of the coronary arteries in the Nephrotic Syndrome of 20 necropsy patients aged 15 to 35 years to determine if coronary atherosclerosis is accelerated.

Project Description: This report describes the status of the major extramural (epicardial) coronary arteries in 20 patients (average age = 24 years) with the nephrotic syndrome (from systemic lupus erythematosus in 13 patients, diabetes mellitus in four, and chronic idiopathic glomerulonephritis in three) and compares the clinical and morphologic observations to those in 14 control subjects (average age = 29 years). Both nephrotic syndrome patients and control subjects ranged in age from 15 to 39 years. The 20 patients with the nephrotic syndrome had significantly more coronary luminal narrowing by atherosclerotic plaques than did the control subjects. The lumens of one or more of the four major coronary arteies were narrowed >75% in cross-sectional area in 8 (40%) of the 20 nephrotic patients and in none of the 14 control subjects. This difference in degree of coronary disease was even more striking when the percent of narrowing in the entire coronary tree was examined: of the 290.5 cm of coronary artery examined in the 20 nephrtoic patients the lumen in 88 cm (30%) was > 50% narrowed in cross-sectional area, whereas of 288 cm of coronary artery examined in the 14 control subjects the lumen in only 5.5 cm (2%) was narrowed to this degree.

Although the cause of the accelerated coronary atherosclerosis in our nephrotic patients is uncertain, hypercholesterolemia, systemic hypertension, corticosteroid therapy and increased clotting tendencies probably all contributed to this acceleration.

Publications: Curry, R. C., and Roberts, W. C.: Status of the Coronary Arteries in the Nephrotic Syndrome - Analysis of 20 Necropsy Patients Aged 15 to 35 Years to Determine If Coronary Atherosclerosis Is Accelerated. American Journal of Medicine (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03061-01 PA

PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

The Extramural and Intramural Coronary Arteries in Juvenile Diabetes Mellitus.

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

PI: Frederick V. Crall, Jr., M.D. — Visiting Medical Student, Univ. of Cincinnati, Ohio

Other: William C. Roberts, M.D. — Chief, PA NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	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)

This report describes the status of the coronary arteries in nine patients with juvenile onset diabetes mellitus and compares the clinical and morphologic observations in them to those in control subjects.

951

Project Description: This report describes the status of the coronary arteries in nine patients (average age = 29 years) with juvenile onset diabetes mellitus (average age onset = nine years), and compares the clinical and morphologic observations in them to those in nine control subjects (average age = 29 years). The nine patients with juvenile diabetes had significantly more extramural coronary luminal narrowing by atherosclerotic plaques than did the control subjects. The lumens of one or more of the four major epicardial coronary arteries were narrowed greater than 75% in cross-ssectioned area in six of the diabetic patients and in none of the nine control subjects. This difference in degree of narrowing of the epicardial coronary arteries was even more striking when the percent of narrowing of the entire lengths of the four major coronary arteries was examined: of the 191 cm of major coronary arterty examined in the nine diabetic patients, the lumen in 90.5 cm (47%) was greater than 50% narrowed in cross-sectioned area, whereas of 155 cm of coronary artery examined in the nine control subjects, the lumen of only two cm (1%) was narrowed to this degree.

Minor degrees of intimal fibrous proliferation, considered of no functional consequence, were observed in the intramural coronary arteriesin the ventricles (excluding papilllary muscle) in six of the nine diabetic patients but in none of the nine control subjects. Periodic acid-Schiff positive material was more frequent and of greater intensity in the diabetic patients (all nine) than in the control subjects (four of nine).

Publications: Crall, F.V. and Roberts, W.C.: The Extramural and Intramural Coronary Arteries in Juvenile Diabetes Mellitus - Analysis of Nine Necropsy Patients Aged 19 to 38 Years With Onset of Diabetes Before Age 15 Years. American Journal of Medicine (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03062-01 PA

PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)
Coronary Anastomotic Sites of Aortocoronary Bypass Grafts

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

PI:	Thomas L. Spray, M.D.	Staff Assoc.	PA	NHLBI
Other:	William C. Roberts, M.D.	Chief	Pa	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Naryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	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)

This study summarizes a status of saphenous vein bypass grafts at and distal to the sites of coronary anastomosis in patients with aortocoronary bypass conduits.

753

PHS-6040
(Rev. 10-76)

Project No. Z01 HL 03062-01 PA

Project Description: The status of the native coronary arteries at necropsy in the vicinity of the coronary anastomoses of saphenous vein aorto-coronary bypass grafts in 20 patients with severe coronary heart disease is presented. Of the 37 graft systems (graft plus coronary artery into which graft inserted) analyzed, the lumina of 44% of the native coronary arteries within the first 2 cm distal to the anastomoses were >75% narrowed in cross-sectional area by atherosclerotic plaques, and the native coronary artery at the site of the anastomoses plaque in 25% of the graft systems. The mean coronary arterial size distal to the site of the coronary graft anastomosis, even after correction for heart weight, was greater in the 13 men than in the 7 women. The residual liminal area squared per gram of heart wieght, however, were similar in both men and women. These results suggest result that: 1) relative coronary vessel size is greater in men then wowen; 2) the luminal area squared per gram myocardial mass (a relative estimation of flow) is the same in the two gruops of patients; and 3) less atherosclerotic plaque is necessary in woemn then in men to produce similar limitation to coronary flow. Thus, vessel size alone cannot account for the higher reported frequency of unsuccessful aorto-coronary bypass porcedures in women.

Publications:

Spray, T.L. and Roberts, W.C.: Status of the Grafts and the Native Coroanry Arteries Proximal and Distal to Coronary Anastomotic Sites of Aroto-Coronary Bypass Grafts. Circulation, 55: 741-749, May 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03063-01 PA

PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Changes in Saphenous Veins Used as Aorto-Coronary Bypass Grafts

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

PI:	Thomas L. Spray, M.D.	Staff Associate	PA	NHLBI
Other:	William C. Roberts, M.D.	Chief	PA	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	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)

This study describes morphologic changes in saphenous veins used as aorto-coronary bypass conduits, and discussed the relative contribution of various factors to these changes. The 3 primary changes are: 1) medial fibrous replacement; 2) adventitial fibrous proliferation; and 3) intimal proliferation. The probable mechanisms how these changes come about were studied.

955

PHS-6040
(Rev. 10-76)

Project No. ZO1 HL 030.63-01 PA

Project Description: This report describes morphologic changes in saphenous veins used as aortocoronary bypass conduits, and discusses the relative contributions of various factors to these changes. The 3 primary changes are: 1) medial fibrous replacement; 2) adventitial fibrous proliferation, and 3) intimal fibrous proliferation. Medial fibrous replacement is caused by vein wall ischemia with loss of smooth muscle cells; advential fibrous proliferation is the result of organization of fibrin deposits and repair of ischemic injury; and intimal fibrous proliferation results form some stimulus, probably fibrin deposition on injured intima, which causes stimulation of smooth muscle cells to become fibroblasts or "myointimal cells." Although all grafts show some changes, the degree and severity of these 3 changes is variable along the length of the grafts and among separate grafts in the same patient.

Publications: Spray, T. L., and Roberts, W. C. : Changes in Saphenous Veins Used as Aorto-Coronary Bypass Grafts. American Heart Journal. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03064-01 PA

PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Medial Calcinosis of Monckeberg

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

PI:	Anthony S. Lachman, M.D.	Visiting Scientist	PA NHLBI
Other:	Thomas L. Spray, M.D.	Staff Assoc.	PA NHLBI
	Donald M. Kerwin, M.D.	Dept. of Path. Georgetown University, Washington, D.C.	
	Gerald I. Shugoll, M.D.	Dept. of Path. Georgetown University Washington, D.C.	
	William C. Roberts, M.D.	Chief,	PA NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	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)

This study describes massive medial calcific deposits in the peripheral and visceral arteries and similar but smaller sized deposits in the coronary arteries of a relatively young woman. Medial calcinosis in the visceral and coronary arteries is extremely rare.

957

Project No. Z01 HL 03064-01 PA

Project Description: Massive medial calcific deposits (Monckeberg's calcinosis) are described in the peripheral and visceral arteries and similar but small-sized deposits in the coronary arteries of a 41-year-old woman with diabetes mellitus. Although observed by roentgenogram fairly commonly during life in the muscular arteries of the legs in middle-aged men, medial calcinosis infrequently involves the visceral arteries and has never, to our knowledge, been documented in the coronary arteries. Although it may be associated with intimal atherosclerosis, medial calcinosis, per se, does not obstruct the lumens of the arteries and, therefore, does not lead to symptoms or signs of limb or organ ischemia. The cause of medial calcinosis remains a mystery but it appears to affect diabetics more frequently than non-diabetics.

Publications: Lachman, A.S., Spray, T.L., Kerwin, D.M., Shugoll, G.I., and Roberts, W.C. : Medial Calcinosis of Monckeberg- A Review of the Problem and a Description of a Patient with Involvement of Peripheral, Visceral and Coronary Arteries. American Journal of Medicine (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03065-01 PA

PERIOD COVERED
July 1, 176 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Morphologic Observations in Biologic Conduits Between Aorta and Coronary Artery

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

PI:	Thomas L. Spray, M.D.	Staff Assoc.	PA	NHLBI
Other:	William C. Roberts, M.D.	Chief	PA	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	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)

This study summarizes structural alterations in aorto-coronary bypass conduits used between aorta and coronary artery in patients as a treatment for ischemic heart disease.

959

Project Description: This report describes morphologic observations in autogenous saphenous veins, autogenous internal mammary arteries, radial arteries and arterial heterografts used as aortocoronary bypass conduits. The normal or expected changes and the abnormal or unexpected changes observed in each type of bypass conduits are discussed. The 3 major changes seen in saphenous vein grafts in the aortocoronary position are: 1) medial fibrosis replacement; 2) adventitial fibrous proliferation; and 3) intimal fibrous proliferation. Medial fibrous replacement is the result of vein wall ischemia and necrosis and ultimately replacement of smooth muscle cells; adventitial fibrosis is the result of organization of fibrin and red cell clot and repair of ischemic damage. The cause of intimal fibrous proliferation is unclear but appears to be the result of chronic repair of injured intima and endothelium. That the severity of the changes varies along the length of one graft or among grafts in the same patient suggests that other factors contribute to the development of the changes observed.

Although some structural changes have been observed internal mammary arteries are relatively resistant to the changes seen in saphenous veins. Radial arterial grafts have shown severe structural changes with time to a degree greater than that seen in the saphenous vein grafts. Heterograft conduits so far have been unsuccessful in the aortocoronary position in humans. The severe changes seen in radial artery and heterograft conduits indicate that they are inadequate for use as substitutes for saphenous vein and mammary artery as bypass conduits from aorta to coronary artery.

Publications: Spray, T. L., and Roberts, W. C.: Morphologic Observations in Biologic Conduits Between Aorta and Coronary Artery. Cardiovascular Clinics (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03066-01 PA

PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Fat Versus FAtigue: Comment on Causes of Atherosclerosis

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

PI:	William C. Roberts, M.D.	Chief	PA	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Nethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	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)

This report serves as a rebuttal to a paper which appeared in Cardiovascular Medicine, March 1977 by Professor Stehbens from Wellington, New Zealand.

961

Project No. Z01 HL 03066-01 PA

Project Description: This rebuttal makes arguments why cholesterol is important in causing atherosclerosis.

Publications: Roberts, W.C.: Fat Versus Fatigue: Comments on Causes of Atherosclerosis. Cardiovascular Medicine, 2: 593-598, June 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03067-01 PA

PERIOD COVERED

July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Coronary 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, M.D.	Chief	PA	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Pathology Branch

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	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)

This piece reviews the status of the coronary arteries in patients dying of coronary heart disease. Emphasis is placed on the diffuse nature of the coronary atherosclerotic plaques, and the possibility of reversing these plaques to some extent by depletion of the lipid component of the plaques.

963

Project Description: The coronary arteries in patients with fatal coronary heart disease may be characterized as follows: 1) The coronary arteries are diffusley involved by atherosclerotic plaques. 2) With few exceptions, the limina of at least teo of the three major coronary arteries are reduced more than 75% in cross-sectional area by atherosclerotic plaques. 3) The atherosclerosis is limited to the epicardial coronary arteries. 4) Certain portions of the coronary tree tendto develop larger atherosclerotic plaques and, therefore, more liminal narrowing than other portions. 5) Of the three types of atherosclerotic plaques, only the complicated ones cause significant (more than 75%) coronary luminal narrowing. 6) The degree of both plaque formation and, in turn, coronary luminal narrowing is similar regardless of the type of fatal coronary event. 7) The comparison of coronary atherosclerotic plaques and the degree of coronary luminal narrowing appear to be similar whether the blood likoprotein pattern was normal or abnormal. 8) The shapes of lumina of atherosclerotic coronary arteries are quite variable. 9) The coronary artery responsible for perfusing an area of myocardium that has become either necrotic or fibrotic is not necessarily the most narrowed (by atherosclerotic plaque) of the three major extramural coronary arteries. 10) Advanced age does not necessarily indicate the presence of severe coronary atherosclerosis.

Among patients with fatal CHD certain observations regarding coronary thrombosis are becoming established. For example, coronary thrombi
- are found in only about 10% of patients who die suddenly.
- are rare in patients with isolated subendocardial infarcts.
- occur in about 60% of patients with fatal transmural acute myocardial infarction.
- usually occur only in the coronary artery responsible for perfusing the area of myocardial necrosis.
- occur in arteries that already are severely narrowed by old atherosclerotic plaques.
-are usually single, occlusive, short, and located entirely in the major trunks.
- Finaaly, the larger the area of myocardial necrosis, the greater the likelihood of coronary thrombosis.

Several factors suggest that coronary thrombosis is a consequence of rather than the precipitating cause of AMI. Slow blood flow and sufficent time are prerequisites for thrombus formation.

Several observations suggest that coronary thrombosis plays a major role in the development of coronary atherosclerosis in the first place.

Among the nonthrombotic acute coronary lesion, only hemorrhages into atherosclerotic plaques are common but there is no evidence that they cause coronary narrowing.

Among patients with myocardial infarction and angiographically normal coronary arteries, an angiographically normal coronary arterial tree has never been demonstrated at the time of AMI; the angiograms were performed after healing of, rather than during, the AMI. Although there are several explanations for

the occurrence of AMI with an angiographically normal coronary artery and although each may be applicable on occasion, the most reasonable appears to be acute coronary embolism with subsequent clot lysis, retraction, or recanalization, which leads to a "normal" appearance on angiography.

Comparisons between methods of examining a coronary artery by selective angiography during life and by histology after death indicate that reduction in liminal diameter by angiography is generally underestimated because coronary atherosclerosis is diffuse in patients with symptomatic CHD.

Publications: Roberts, W.C.: Coronary Heart Disease: A Review of Abnormalities Observed in the Coronary Arteries. Cardiovascular Medicine, Vol.2 No.1 29- 49, January 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03068-01 PA

PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Aschoff Bodies in Operatively Excised Atrial Appendages and in Papillary Muscles.

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

PI:	Renu Virmani, M.D.	Guest Worker	PA	NHLBI
Other:	William C. Roberts, M.D.	Chief	PA	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	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)

This study summarizes the frequency of Aschoff bodies in atrial appendages and papillary muscles in patients undergoing mitral valve operations. It also characterizes the hemodynamic mitral lesions.

PHS-6040
(Rev. 10-76)

Project Description: Among 481 patients undergoing various mitral valve operations, Aschoff bodies were found in 40 (21%) of 191 operativley excised left atrial appendages, in four (2%) of 273 operativley excised left ventricular papillary muscles (1 percent), and in one (6%) of 17 patients with both left atrial appendage and papillary muscle operativley excised. Of the total of 45 patients with Aschoff bodies, 44 preoperativley had mitral stneosis, and only one, a 10-year-old boy, had pure mitral regurgitation. Sinus rhythm was present in 38 (84%), and atrial fibrillation in seven (16%). Perioperativley, only one of the 45 patients with Aschoff bodies had clinical or laboratory stigmata compatible with acute rheumatic fever, and 58% had an illness compatible with acute rheumatic fever at any time.

Publications: Virmani, R. and Roberts, W.C.: Aschoff Bodies in Operativley Excised Atrial Appendages and in Papillary Muscles. Circulation, 55: 559-563, April 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03069-01 PA

PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Aschoff Bodies At Necropsy In Valvular 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, MD.	Chief	PA	NHLBI
Other:	Renu Virmani, M.D.	Guest Worker	PA	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	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)

This report summarizes the frequency of Aschoff bodies in a large group of patients over 14 years of age with severe valvular heart disease.

968

PHS-6040
(Rev. 10-76)

Project Description: Among 543 necropsy patients over age 14 years with severe chronic valvular heart disease, Aschoff bodies were found in 11 patients (2%). The ages of the 11 patients ranged from 18 to 68 years (avg. 38), and nine had had a history of acute rheumatic fever eariler in life. Clinically, nine of the 11 patients had mitral stenosis with or without other valvular lesions, one had isolated aortic regurgitation and one had mitral and oartic regurgitation. All 11 patients had diffuse fibrous thickening of the mitral valve leaflets. Aschoff bodies are the only anatomic lesion pathognomonic of rheumatic heart disease, they are indicative of diffuse anatomic disease of the mitral leaflets, and they usually indicate the presence of mitral stenosis.

Publications: Roberts,W.C. and Virmani, R.: Aschoff Bodies At Necropsy In Valvular Heart Disease - An Analysis of 543 Patients Over 14 Years of Age. Circulation (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03070-01 PA

PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

"Mitral Stenosis" Secondary to "massive" Mitral Annular Calcific Deposits

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

PI:	Antonio C. deLeon, Jr., M.D.	Div. of Card., Dept. of Medicine Georgetown Univ. Med. Cen. Wash., D.C.
Other:	William J. Hammer, M.D.	Div. of Card., Dept. of Medicine Georgetown Univ. Med. Cen. Wash., D.C.
	William C. Roberts, M.D.	Chief, PA NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	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)

This study summarizes observations in 4 patients with physiologic documentation of mitral stenosis secondary to calcific deposits in the mitral anulus but an otherwise normal mitral valve.

970

Project Description: Certain observations are described in 4 elderly women with massive mitral anular calcific deposits, small thick-walled left ventricles and diastolic gradients between pulmonary artery wedge position (or left atrium) and left ventricle. All 4 patients had some degree of obstruction to left ventricular outflow. Examination at necropsy (2 patients) or at operation (2 patients) disclosed only focal fibrous thickening of the mitral leaflets without commissural or chordal fusion. By auscultation, none had mitral opening snaps, only 2 had loud first heart soundsand only 1 had a mitral diastolic rumble. Hemodynamic documentation of a diastolic gradient between pulmonary artery wedge position (or left atrium) and left ventricle in the presence of massive mitral anulus calcific deposits and in the absence of diffuse disease of the mitral leaflets has not been demonstrated previously. The distolic gradients are considered to result from the combination of the large mitral anular calcific deposits and the small, thick walled, non-compliant left ventricles.

Publications: deLeon, A.C., Hammer, W.J., and Roberts, W.C.: "Mitral Stenosis" Secondary to "Massive" Mitral Anular Calcific Deposits and Small, Hypertrophied Left Ventricles- Hemodynamic Documentation in Four Patients.American Journal of Medicine (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03071-01 PA

PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Healed Left-sided Infective Endocarditis

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

PI; William C. Roberts, M.D. Chief, PA NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	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)

This report summarizes clinical and morphologic observations in 59 patients who had positive histories of active left-sided infective endocarditis which had been eradicated by antibiotic therapy.

972

PHS-6040
(Rev. 10-76)

Project Description: Clinical and morphologic observations are described in 59 patients who had histories of active left-sided endocarditis which had been eradicated by antibiotic therapy. Of the 59 patients, 42 were from a group of 584 necropsy patients with fatal valvular heart disease of various types: the remaining 17 were from a group of 79 patients who had undergone mitral or aortic valve replacement or both because of severe mitral or aortic regurgitation or both. Of the 59 patients, examination of their heart at necropsy, (42 patients) or at valve replacement (17 patients) disclosed that 30 (51 percent) had anatomic lesions which could readily be attributed to the active infective endocarditis which healed: cuspal perforation in 16 patients; rupture of chordae tendineae in 15, and aneurysms at or near the involved valve in 3. Unequivocal residua of the valvular infection were more common in the purely incompetent than in the stenotic cardiac valves. Comparsion of observations in the 42 necropsy patients with healed left-sided infective endocarditis to observations (previously reported) in 74 necropsy patients with active left-sided infective endocarditis showed that among the patients with healed endocaridtis the infection more commonly involved a previously abnormal valve, that the causative organism was more likely to be alpha streptococcus, and that recognized predisposing factors were less frequent.

Publications: Roberts,W.C. and Buchbinder, N.A.: Healed Left-sided Infective Endocarditis: A Clinico-pathologic Study of 59 Patients. American Journal of Cardiology (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03072-01 PA

PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Characteristics and Consequences of Infective Endocarditis

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

PI: William C. Roberts, M.D. Chief, PA NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	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)

This report summarizes morphologic observations over a number of years in patients with active or healed infective endocarditis.

974

PHS-6040
(Rev. 10-76)

Project No. Z01 HL 03072-01 PA

Project Desription: This report summarizes studies carried out during the past 15 years in this unit on nearly 150 patients with fatal active infective endocarditis and 59 patients with histories of active infective endocarditis which subsequently healed. Study of these patients who all died after the introduction of antibiotics clearly shows that today infection on a cardiac valve is fair more common in a previously normal valve than one which is previosusly abnormal. Among patients with previously abnormal valves, however, the possibility of acquiring an infection on that valve however is greater than in patients with previously normal valves. Because there are more previously normal valves however, in people than abnormal ones, more patients with infection develop that infection a previously normal valve. It is clear from study of these patients that infection on a previously normal valve is far more dangerous than is infection on a previously scarred valve. A previously scarred valve is much harder to destroy by the infective process than is the fragile thin normal valve. Also the studies here in patients with healed infective endocarditis shows that only about 1/3 of the patients with active infective endocarditis who survive the infection because of antiobiotic therapy actually are left with a functionally normal valve.

Publications: Roberts, W.C.: Characteristics and Consequences of Infective Endocarditis (Active or Healed or Both) As Learned From Morphologic Studies. Chapter in book entitled: Infective Endocarditis, published by Grune & Stratton in 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03073-01 PA

PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Structural Changes in Porcine Xenographs Used As Subsitute Cardiac Valves

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

PI:	Thomas L. Spray, M.D.	Staff Associate	PA	NHLBI
Other:	William C. Roberts, M.D.	Chief	PA	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	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)

This study describes gross and histologic observation in 51 Hancock glutaraldehyde-preserved porcine heterograft bioprostheses from 41 patients.

976

PHS-6040
(Rev. 10-76)

Project Description: Gross and histologic observations are desribed in 51 Hancock glutaraldehyde-preserved porcine heterograft bioprostheses from 41 patients: 33 valves from 25 patients had been in place for <2 months (early) and 18 valves from 17 patients were examined at later periods up to 75 months (late) after implantation. The major gross changes were cuspal thrombosis (5 bioprostheses) and cuspal degeneration (3 bioprostheses). Major histologic changes observed in 44 bioprostheses (26 early and 18 late) examined histologically were: 1) fibrin deposits on inflow & outflow surfaces of the cusps; 2) inflammatory cell infiltrates; 3) histiocyte deposition; 4) giant cell formation, and 5) focal disruption of the fibrocollagenous structure of the cusps. These observations indicate that porcine bioprostheses are not biologically inert in the human circulation. Valve failure, however, is rare at the implantation periods studied.

Publications: Spray, T. L., and Roberts, W. C. : Structural Changes In Procine Xenografts Used As Substitute Cardiac Valves- Gross and Histologic Observations in 51 Glutaraldehyde-Preserved Hancock Valves in 41 Patients. American Journal of Cardiology (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03074-01 PA

PERIOD COVERED

July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Aortic Dissection After Aortic Replacement

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

PI:	Walinjom F. Muna, M.D., Ph.D.	Visiting Fellow	PA	NHLBI
Other:	Thomas L. Spray, M.D.	Staff Assoc.	PA	NHLBI
	Andrew G. Morrow, M.D.	Chief	SU	NHLBI
	William C. Roberts, M.D.	Chief	PA	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Pathology Branch

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	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)

This report calls attention to dissection of the aorta after aortic valve replacement by briefly describing certain clinical and necropsy findings in these 2 patients.

978

PHS-6040
(Rev. 10-76)

Project No. Z01 HL 03074-01 PA

Project Description: Dissection of the aorta is a well-known occurrence in certain patients with aortic valve disease. Its occurrence, however, after aortic valve replacement is not well known, and it occurrence in patients with valvular aortic stenosis, in contrast to patients with pure aortic regurgitation, also is not widely appreciated. Among 847 patients in whom aortic valve replacement has been carried out at the National Heart, Lung, and Blood Institute in the past 16 years, aortic dissection was known to occur postoperatively in 2 of them. This report calls attention to this complication after aortic vavle replacement by briefly describing certain clinical and necropsy findings in these 2 patients.

Publications:

Muna, W.F., Spray, T.L., Morrow, A.G., and Roberts, W.C.: Aortic Dissection After Aortic Valve Replacement in Patients With Valvular Arotic Stenosis. Journal of Thoracic and Cardiovascular Surgery, 74:65-69, July 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03075-01 PA

PERIOD COVERED

July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Echocardiographic Observations in Opiate Addicts With Active Infective Endocarditis.

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

PI:	Joseph J. Andy, M.D.	Dept. of Medicine	D.C. General Hospital
Other:	Mazhar U. Sheikh, M.D.	Dept. of Medicine	D.C. General Hospital
	Nayab Ali, M.D.	Dept. of Medicine	D.C. General Hospital
	Boisey O. Basrnes, M.D.	Dept. of Medicine	D.C. General Hospital
	Lay M. Fox, M.D.	Dept. of Medicine	D.C. General Hospital
	Charles L. Curry, M.D.	Dept. of Medicine	Howard Univ., Wash.,D.C.
	William C. Roberts, M.D.	Chief, PA	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Pathology Branch

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	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)

Echocardiographic observations are described in 25 opiate addicts with active infective endocarditis (IE) involving apparently previously normal vavles.

980

PHS-6040
(Rev. 10-76)

Project Description: Echocardiographic observations are described in 25 opiate addicts with active infective endocarditis (IE) involving apparently previuosly normal valves. Eleven patients had isolated tricuspid-valve IE, 7 had both right (tricuspid valve) and left sided IE, and 7 had isolated left-sided IE (mitral valve in 6). Twenty patients (80 percent) had tricuspid-valve regurgitation, 12 had mitral regurgitation, 3 had aortic regurgitation, and none had pulmonic regurgitation. Of the 75 cardiac valves (excluding the pulmonic) in the 25 patients, echocardiographic abnormalities consistent with active IE were detected in 26 (74 percent) of the 35 clinically incompetent valves but in none of the 40 competent valves. Comparison of the 20 dysfunctioning tricuspid valves to the 12 incompetent mitral valves indicated that the echocardiogram was less sensitive in detecting tricuspid valves lesions, that rupture of tricuspid valve chordae tendineae was absent or not detectable, and that tricuspid valve vegetations tended to be larger.

Publications: WCR# 273

Andy, J.J., Sheikh, M.U., Ali, N., Barnes, B.O., Fox, L.M., Curry, C.L., and Roberts, W.C.: Echocardiographic Observations in Opiate Addicts with Active Infective Endocarditis. Frequency of involvement of the various valves and comparison of echocardiographic features of right and left-sided cardiac valve endocarditis. American Journal of Cardiology 40:17-23, July 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03076-01 PA

PERIOD COVERED

July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Prosthetic Cardiac Valves: A Comparison of the Four Basic Desings

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

PI:	Thomas L. Spray, M.D.	Staff Assoc.	PA	NHLBI
Other:	William C. Roberts, M.D.	Chief	PA	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Pathology Branch

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	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)

This piece summarizes the various advantages and disadvantages of the four presently commonly used prosthetic cardaic valves.

982

Project No. Z01 HL 03076-01 PA

Project Description: This report summarizes certain advantages and disadvantages of the four presently commonly used cardiac valves. These include the caged-ball, caged-disc, tilting-disc and porcine prosthesis. Emphasis is placed on the advantages of the tilting-disc type prosthesis and the porcine prosthesis although it is pointed out that sufficent time, i.e. greater than 5 years, is not available in a large number of patients and therefore, a long-term judgement on the porcine prosthesis must be reserved at present.

Publications: Spray, T.L., and Roberts, W.C.: Prosthetic Cardiac Valves: A Comparison of the Four Basic Designs. Will appear in a book entitled: Special Aspects of Surgery of Rheumatic Heart Disease in Children. Edited by M. Gotsman and J.B. Rorman. To be published in 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03077 -01 PA

PERIOD COVERED

July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Severe Aortic Regurgitation Secondary to Idiopathic Aortitis

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

PI:	Howard S. Honig, M.D.	Dept. of Medicine, Georgetown Univ. Med. Center, Washington, D.C.
Other:	Alan M. Weintraub, M.D.	Dept. of Medicine, Georgetown Univ. Med Center, Washington, D.C.
	Mario N. Gomes, M.D.	Dept. of Surgery, Georgetown Univ. Medical Center, Washington, D.C.
	Charles A. Hufnagel,M.D.	Dept. of Surgery, Georgetown Univ. Medical Center, Washington, D.C.
	William C. Roberts, M.D.	Chief, PA NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Pathology Branch

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	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)

Clinical and morphologic features are described in two young adults with aortic regurgitation secondary to chronic aortitis. A case is made for the aortitis to be of the Takayasu's variety.

984

Project Description: Clinical and morphologic features are described in two relatively young adults with aortic regurgitation secondary to chronic aortitis. The regurgitation in each was severe enough to require aortic valve replacement. Both patients had normchromic, normocytic anemia, considerable weight loss despite congestive cardiac failure, and negative serologic tests for syphilis. These systemic manifestations in association with the aortitis suggests that both had Takayasu's arteritis. In addition, one patient had total occlusion at the origin of one subcalvian artery (classic pulseless disease). Takayasu's arteritis must be added to the list of causes of severe aortic regurgitation.

Publications: Honig, H.S., Weintraub, A.M., Gomes, M.N., Hufnagel, C.A. and Roberts, W.C.: Severe Aortic Regurgitation Secondary to Idiopathic Aortitis: A Maifestation of Takayasu's Arteritis. American Journal of Medicine (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03078-01 PA

PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

A Hitherto Undescribed Cause of Prosthetic Mitral-Valve Obstruction

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

PI:	Ancil A. Jones, M.D.	Clinical Assoc.	PA	NHLBI
Other:	John B. Otis, M.D.	Dept. of Path., Georgia Baptist Hosp. Atlanta, Georgia		
	Gerald F. Fletcher, M.D.	Dept. of Path., Georgia Baptist Hosp. Atlanta, Georgia		
	William C. Roberts, M.D.	Chief,	PA	NHLBI

COOPERATING UNITS (if any)
Dept. of Pathology & Medicine, Georgia Baptist Hospital, Atlanta, Georgia

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	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)

This study describes a patient with severe prosthetic mitral-valve obstruction produced by entanglement of sutures across the central axis of the prosthesis on its ventricular side.

986

PHS-6040
(Rev. 10-76)

Project Description: A patient is described in whom severe prosthetic mitral valve obstruction was produced by entanglement of sutures across the central axis of the prosthesis on its ventricular side. The entangled sutures prevented the tilting disc occluder from falling into the left ventricular cavity during ventricular diastole.

Publications:

Jones, A.A., Otis, J.B., Fletcher, G.F., and Roberts, W.C.: A Hitherto Undescribed Cause of Prosthetic Mitral-Valve Obstruction. Journal of Thoracic and Cardiovascular Surgery (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03079-01 PA

PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Prosthetic Valve Endocaridtis Due to Listeria Monocytogenes

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

PI: William C. Roberts, M.D. Chief, PA NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	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)

This study describes a patient with prosthetic valve endocarditis due to listeria monocytogenes.

988

PHS-6040
(Rev. 10-76)

Project No. Z01 HL 03079-01 PA

Project Description: Prosthetic valve endocarditis has been caused by a variety of unusual organisms but no reports have appeared of its being caused by Listeria monocytogenes. Such was a case however, in a patient studied by us and described in this report.

Publications: Breyer, R.H., Arnett, E.N., and Roberts, W.C.: Prosthetic Valve Endocarditis Due to Listeria Monocytogenes. American Journal of Clinical Pathology (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03080-01 PA

PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Sarcoidosis of the Heart

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

PI:	William C. Roberts, M.D.	Chief, PA NHLBI
Other:	Hugh A. McAllister, M.D.	Chief, Dept. of Pathology, Armed Forces Inst. of Path., Washington, D.C.
	Victor J. Ferrans, MD.	Chief, Ultrastructure PA NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	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)

This study summarizes clinical and necropsy observations in 35 patients with sarcoid granulomas in their hearts at necropsy.

990

PHS-6040
(Rev. 10-76)

Project Description: Certain clinical and morphologic observations are described in 35 necropsy patients with cardiac sarcoidosis and similar observations are summarized in 78 previously reported necropsy patients with cardiac sarcoidosis. All patients had non-necrotic ("hard") granulomas in lymph nodes and either carcoid granulomas in the heart (109 patients) or transmural myocardial scarring (4 patients) unassociated with coronary arterial narrowing, i.e., healed granulomas. The 113 patients analyzed were divied into 2 major groups: 1) those in whom cardiac dysfunction,if present, was clearly the result of sarcoid granulomatous infiltration of the heart (89 patients), and 2) those in whom cardiac dysfunction, if present, was clearly not the result of sarcoid involvement of the heart (24 patients).

Analysis of the 89 group I patients discloed that death was sudden (arrhythmia) in 60 (67%), secondary to progressive congestive cardiac failure in 20 (23%), from recurring pericardial effusion in 3 (3%), and from other or unknown causes in 6 (7%). Sudden death was the initial manifestation of sarcoidosis in 10 (17%) of the 60 patients dying suddenly. Other than premature ventricular beats, ventricular tachycardia was the most common arrhythmia (17 patients), and complete heart block was the most common conduction disturbance (25 patients). Complete bundle branch block occurred in 21 patients. Ventricular aneurysm 8 patients), and papillary muscle dysfunction (possibly 16 patienta) were other caridac disturbances observed.

Although corticosteroid therapy tends to cause fibrous replacement of myocardial sarcoid granlumoas, a possible consequence of this medication is the development of ventricular aneurysm. Only one of the 8 patients with cardiac aneurysm had not received corticosteroid therapy; of the 9 patients who received this medication for longer than 1 month, 4 had no residual myocardial scars and the other 5 had extensive myocardial scarring in addition to some residual granlumoas. Sarcoid granulomas in the heart appear to be more responsive to corticosteroid therapy than are sarcoid granulomas located in other body organs.

Most patients with cardiac sarcoidosis causing dysfunction (Group I) present intially with manifestations related to the heart. Furthermore, most patients with cardiac sarcoidosis have little or no clinical evidence of dysfunction of an organ system other than the heart. Usually the course in patients with extensive cardiac sarcoidosis is not prolonged. The length of symptoms of cardiac dysfunction from cardiac sarcoidosis was 12 12 months or less in 63% of the patients in whom this information was available.

Sarcoidosis appears to be the major cause of granulomas (giant-cell) myocarditis. Most patients reported as having idiopathic granulomatous myocarditis probably have cardiac sarcoidosis but granulomatous involvement of other organs, particularly lymph nodes, had been overlooked.

Publications: Roberts, W.C., McAllister, H.A. and Ferrans, V.J.: Sarcoidosis of the Heart. The American Journal of Medicine, 63: 86-108, July 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03081-01 PA

PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Trichinosis Causing Extensive Ventricular Mural Endocarditis

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

PI:	Joseph J. Andy, M.D.	Dept. of Medicine, Howard Univ. Wash., D.C.
Other:	Joseph P. O'Connell,M.D.	Dept. of Pathology, Surburban Hos. Beth.,Md.
	Robert C. Daddario,M.D.	Dept. of Medicine, Surburban Hos. Beth., Md.
	William C. Roberts, M.D.	Chief, PA NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	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)

Clinical and morphologic observations are described in a woman with fatal trichinosis.

992

PHS-6040
(Rev. 10-76)

Project No. Z01 HL 03081-01 PA

Project Description: Clinical and morphologic observations are described in a 46-year-old woman with fatal trichinosis. Attention is called to the occurrence of extensive ventricular endocardial damage with superimposed thrombosis. Evidence is presented to indicate that the most likely cause of the endocardial damage is the associated eosinophilia. The mechanism by which eosinophils damage endocardium, however, remains obscure.

Publications: Andy, J.J., O'Connell, J.P., Daddario, R.C. and Roberts, W.C. Trichinosis Causing Extensive Ventricular Mural Endocarditis With Superimposed Thrombosis - Evidence that Severe Eosinophilia Damages Endocardium. American Journal of Medicine (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03082-01 PA

PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)
Cardiomyopathy and Myocarditis: Morphologic Features

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

PI: William C. Roberts, M.D. Chief, PA NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	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)

This report describes certain morphologic changes observed at necropsy in patients with various non-inflammatory and inflammatory heart muscle diseases.

994

PHS-6040
(Rev. 10-76)

Project Description: This report summarizes primarily morphologic observations in a number of non-inflammatory cariodmyopathies incldluing the idiopathic dilated and hypertrophic types, endomyocardial disease with and without eosinophilia and the infiltrative types. In addition, it describes certain inflammatory cardiomyopathies, IE myocarditis, but empahsis is placed on the rarity of myocarditis now compared to its extreme frequency a number of years ago. Today the non-inflammatory cardiomyopathies are far more common than are the inflammatory myopathies whereas the reverse appeared to be the situation prior to the introduction of antibiotics in the 1940's.

Publications: Roberts, W.C.: Cardiomyopathy and Myocarditis: Morphologic Features. The Heart, 4th Edition.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03083-01 PA

PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Duchenne's Muscular Dystrophy

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

PI:	Shirley Rubler, M.D.	Cardiovascular Section, Dept. of Medicine University of Pennsylvania, School of Med.
Other:	Joseph K. Perloff, M.D.	Cardiovascular Section, Dept. of Medicine University of Pennsylvania, School of Med.
	William C. Roberts, M.D.	Chief, PA NHLBI

COOPERATING UNITS (if any)
Cardiovascular Section, Dept. of Medicine, University of Pennsylvania School of Medicine, Philadelphia, Penn.

LAB/BRANCH
Pathology Branch

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INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	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)

This report reviews the cardiovascular changes observed in patients with fatal Duchenne's muscular dystrophy.

996

PHS-6040
(Rev. 10-76)

Project Description: This report summarizes cardiovascular observations in three patients with Duchenne's progressive muscular dystrophy. Emphasis is placed on the lack of myocardial scarring the ventricular septum in these patients and the presence of such scarring in the basal portion of left ventricular free wall. The scarring in the left ventricular free wall causes the free wall to be thinner than the ventricular septum and thus Duchenne's progressive dystrophy is a cause of asymmetric hypertrophy. None of these patients however had myocardial fibor disoriéntation so therefore although asymmetric septal hypertrophy was present hypertrophic cardial myopathy is not a part of this condition.

Publications: Rubler, S., Perloff, J. K., and Roberts, W. C. : Duchenne's Muscular Dystorphy. American Heart Journal (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03084-01 PA

PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)
Electrocardiogram in Hematologic and Neoplastic Disorders

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

PI:	Joseph Lindsay, Jr., M.D.	Investigator, Dept. of Medicine, George Washington University Medical Center, Washington D.C.
Other:	Samuel D. Goldberg, M.D.	Investigator, Dept. of Medicine George Washington University Medical Center, Washington, D.C.
	William C. Roberts, M.D.	Chief, PA NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

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INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	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)

Electrocardiographic features observed in patients with various neoplasms involving the heart are described.

998

Project No. Z01 HL 03084-01 PA

Project Description: Summarized here are electrocardiographic manifestations produced by cardiac tumors both primary and secondary. Included herein, are summaries of these findings in patients with myxoma, rhabdomyoma, fibroma, sarcoma and a number of secondary cardiac neoplasms.

Publications: Lindsay, J.L., Goldberg, S.D. and Roberts, W.C.: Electrocardiogram in Hematologic and Neoplastic Disorders. Cardiovascular Clinics (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03085-01 PA

PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

A Discussion on Hypertrophic Cardiomyopathy

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

PI:	Thomas L. Spray, M.D.	Staff Assoc.	PA	NHLBI
Other:	Barry J. Maron, M.D.	Senior Invest.	CB	NHLBI
	Andrew G. Morrow, M.D.	Chief,	SU	NHLBI
	Stephen E. Epstein, M.D.	Chief,	CB	NHLBI
	William C. Roberts, M.D.	Chief,	PA	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

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INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	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)

This report summarizes certain clinical and morphologic features in patients with hypertrophic cardiomyopathy.

1000

PHS-6040
(Rev. 10-76)

Project No. Z01 03085-01 PA

Project Description: In this report are summarized many of the clinical and morphologic observations learned in the past 15 years at the NHLBI from study of patients with hypertrophic cardiomyopathy. The causes of the various symptoms namely chest pain, syncope or presyncope, exertional dyspnea are discussed. In addition, the association between hypertrophic cardiomyopathy and systemic hypertension is summarized. Also the cause of the precardial murmurs in patients with hypertrophic cardiomyopathy is discussed. Because the right ventricular outflow obstruction in this entity is also summarized. The causes of certain discrepancies between the thicknesses of the ventricular walls measured at necropsy and during life by echocardiogram in this condition is summarized. Also Dr. Morrow summarizes his results with the myotomy-myectomy procedure in this condition.

Publications: Spray, T.L., Maron, B.J., Morrow, A.G., Epstein, S.E. and Roberts, W.C. : A Discussion on Hypertrophic Cardiomyopathy. American Heart Journal (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03086-01 PA

PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)
Aortic Valve Atresia Associated With Ventricular Septal Defect

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

PI:	Lowell W. Perry, M.D.	Div. of Ped. Card., Dept. of Child Health & Development. Children's Hos. National Medical Center, Washington, D.C.
Other:	Lewis P. Scott, III,M.D.	Div. of Ped. Card., Dept. of Child Health & Development. Children's Hos. National Medical Center, Washington, D.C.
	Stepehn R. Shapiro,M.D.	Div. of Ped. Card., Dept. of Child Health & Development. Children's Hos. National Medical Center, Washingon, D.C.
	Roma S. Chandra,MD.	Dept. of Path. Children's Hos. National Medical Center, Washington, D.C.
	William C. Roberts, M.D.	Chief, PA NHLBI

COOPERATING UNITS (if any)
Div. of Pediatric CArdiology, Department of Child Health & Development and Pathology.
Children's Hospital National Medical Center, Washington, D.C.

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	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)

This study summarizes clinical pathologic features in 4 patients with ventricular septal defect associated with aortic valve atresia.

1002

PHS-6040
(Rev. 10-76)

Project No. Z01 HL 03086-01 PA

Project Description: Aortic valve occurs in 2 types: in type I, the left ventricle is minute, the mitral avalve is atretic (typeIA) or hypolastic (type IB), and the ventricular septum is intact; in type II, the left ventricle is weel-developed, the mitral valve is either atretic (type IIA) or normal (type IIB), and 1 or more defects are present in the ventricular septum. In this report, clinical and morphologic observations are described in 4 patients with the type II variety of aortic valve atresia. Useful clues to the diagnosis of type II aortic valve atresia include transmission of a precordial murmur to the back, near normal left ventricular forces on elctrocardiogram in the presence of right ventricular hypertrophy (detectable only by increased R/S ratio in lead V1), and absence of right atrial enlargement. Unless the mitral valve also is atretic, significant shunting at the atrial level is usually absent. Angiography can provide the definite diagnosis by demonstrating a left ventricle, lack of continuity between left ventricle and aorta, and a hypolastic ascending aorta which is filled via the ductus arteriosus. Recognition of the type II variety of aortic valve atresia is important because of the possibility of operative palliation or repair.

Publications: Perry, L. W., Scott, L.P., Shapiro, S.R., Chandra, R.S., and Roberts, W.C.: Aortic Valve Atresia Associated With Ventricular Septal Defect - A Clinicopathologic Study of 4 Newborns. Chest (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03087-01 PA

PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Canine Hearts After 24-Hour Preservation and Orthotopic Transplantation

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

PI:	Thomas L. Spray, M.D.	Staff Associate	PA	NHLBI
Other:	William C. Roberts, M.D.	Chief	PA	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	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)

This study summarizes gross and histologic observations in canine hearts studied after 24-hour preservation and orthotopic transplantation.

100+

Project No. Z01 HL 03087-01 PA

Project Description: Ten dogs underwent orthotopic cardiac transplantation after preservation of the donor heart for 24 hours in an oxygenated hypothermic, hypertonic, intracellular solution, either with (5 dogs) or without (5 dogs) continuous oxygenated low pressure perfusion. Eight dogs survived 24 hours after transplantation at which time they were sacrificied. The 2 non-survivors were among the 5 non-perfused hearts. Examination of all 10 donor hearts showed differences between the 2 groups: 4 of 5 nonperfused hearts showed severe transmural myocardial coagulation necorsis but only small foci of contraction band necrosis, but only minimal and focal coagulation necrosis, indicating less severe nypoxic damage.

These results indicate that oxygenated perfusion with a hypothermic, hypertonic, intracellular solution may permit improved transplant survival after extended cardiac preservation.

Publications: WCR# 271

Spray, T. L., Watson, D. C., and Roberts, W. C. : Morphology of Canine Hearts After 24-Hour Preservation and Orthotopic Transplantation. Journal of Thoracic and Cardiovascular Surgery. 73: 880-886, June 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03088-01 PA

PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Fibrosing Mediastinitis Causing Pulmonary Arterial Hypertension

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

PI:	William C. Roberts, M.D.	Chief	PA	NHLBI
Other:	James M. Bacos, M.D.	Div. of Card.	Wash. Hos. Cen.	Wash., D.C.
	Abe M. Macher, M.D.	Clinical Assoc.		NIAID
	H. Brandis Marsh, M.D.	Div. of Card.	Wash. Hos. Cen.	Wash., D.C.
	Daniel D. Savage, M.D.	Senior Invest.	CB	NHLBI

COOPERATING UNITS (if any)
Division of Cardiology, Dept. of Medicine, Washington Hospital Center, Washington, D.C.

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	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)

This study summarizes clinical and morphologic observations in two patients with severe pulmonary arterial hypertension without pulmonary venous hypertension resulting from fibrosing mediastinitis.

1006

Project No. Z01 HL 03088-01 PA

Project Description: Clinical and morphologic observations are described in two patients with severe pulmonary hypertension without pulmonary venous hypertension from fibrosing mediastinitis (FM). In one patient, both main pulmonary arteries and one major pulmonary vein were severely narrowed by dense fibrous tissue, and in the second patient, only the right main pulmonary artery was severely narrowed. Both patients had normal intrapulmonary arteries and normal pulmonary parenchyma. Of 9 previously described necropsy patients with pulmonary hypertension due to FM, 7 had severe narrowing of multiple large pulmonary veins and in 6 of them the pulmonary hypertension was due entirely to pulmonary venous obstruction. In one patient, the pulmonary hypertension was due to obstruction of one main pulmonary artery and several large pulmonary veins. Each of these 7 reported patients had severe changes in small intrapulmonary arteries. Of the other two previously reported patients with pulmonary hypertension from FM, one had severe narrowing of only the main right pulmonary artery, and the other, of both main pulmonary arteries. Thus, although pulmonary arterial hypertension in patients with FM is usually due to obstruction of multiple large pulmonary veins and to severe secondary changes in small intrapulmonary arteries, FM can cause severe pulmonary hypertension by obstructing the right or both main pulmonary arteries.

Publications: Arnett, E. N., Bacos, J. M., Macher, A. M., Marsh, H. B., Savage, D. D., and Roberts, W. C. : Fibrosing Mediastinitis Causing Pulmonary Arterial Hypertension Without Pulmonary Venous Hypertension. American Journal of Medicine (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03089-01 PA

PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)
Primary Sarcoma of the Pulmonary Trunk and/or Right or Left Main Pulmonary Artery.

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

PI:	Barry M. Shmookler, M.D.	Resident in Pathology, Washington Hos. Center, Washington, D.C.
Other:	H. Brandis Marsh, M.D.	Div. of Cardioology, Dept. of Medicine Washington Hos. Center, Washington, D.C.
	William C. Roberts, M.D.	Chief, PA NHLBI

COOPERATING UNITS (if any)
Dept. of Pathology & Medicine Washington Hospital Center, Washington, D.C.

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	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)
This study describes clinical and morphologic observation in 2 patients with primary sarcoma of the pulmonary trunk and summarizes observations in 35 previously reported patients with this rare neoplasm.

1008

PHS-6040
(Rev. 10-76)

Project No. ZO1 HL 030 89-01 PA

Project Description: Clinical and morphologic observations are described in two women with primary sarcoma of the pulmonary trunk, and observations in 35 previously reported patients with primary sarcoma involving a major extrapulmonary pulmonary artery are summarized. The neoplasm produces symptoms by causing obstruction to right ventricular outflow or by dislodging tumor fragments to the smaller intrapulmonary pulmonary arteries with or without pulmonary infarction. The sarcoma nearly always arises from the pulmonary trunk to which it is firmly attached. Although it grows to a large size within the lumen, it infrequently, despite its highly malignant histologic pattern, extends through the wall of the pulmonary trunk or metastasizes outside the pulmonary circulation. It may mimin a variety of more common disorders. Diagnosis can be achieved by angiography and treatment starts with a total excision.

Publications: Shmookler, B. M., Marsh, H. B., and Roberts, W. C. : Primary Sarcoma of the Pulmonary Trunk and/or Right or Left Main Pulmonary Artery-A Rare Cause of Obstruction to Right Ventricular Outflow: Report of Two Patients and Analysis of 35 Previosuly-Reported Patients. American Journal of Medicine (in press.)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03090-01 PA

PERIOD COVERED
July 1, 1976 - September 30, 1977

TITLE OF PROJECT (80 characters or less)

Examining the Heart at Necropsy

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

PI: William C. Roberts, M.D. Chief, PA NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
175 hrs.	175 hrs.	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)

This piece summarizes methods used in this laboratory during the past 18 years to examine hearts at necropsy.

1010

Project Description: Three principles useful in examination of the heart at necropsy have been presented. Principle #1 is fix the heart in formalin or in some other preserving or firming medium before opening it. Fixation of the specimen before incision allows retention of the 3-dimensional configuration of the heart and consequently more meaningful comparisons between chamber sizes, wall thickness, valve orfice, etc. Principle #2 is x-ray the fixed heart specimen before opening it. Radiographs reduce the 3-dimensional intact heart to a 2-dimensional structure and provide additional means of visualizing chamber sizes and wall thicknesses. Principle #3 is the method chosen to open the heart is determined by the type of cardiac disease present or suspected. There is no one way to open the heart, and, in general, hearts with different disease require different methods of opening. Opening hearts according to the flow of blood, a common practice, is one of the least desirable methods of incising hearts.

Publications: Roberts, W.C.: Examining the Heart at Necropsy. Chapter in book entitled: Cardiac Radiology to be published by C.V. Mosby, St. Louis in 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 030 91-01 PA

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Human Myocardial Structure in 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 IR PA NHLBI
Other: None

COOPERATING UNITS (if any)

None

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.05	0.05

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)

This project consisted of a review of cardiac morphologic changes observed in a total of 111 patients with heart diseases of various causes. Based on this review, criteria were formulated for the definition of degenerative changes in cardiac muscle cells.

1012

Project Description: Degenerative changes observed in biopsies of ventricular myocardium from 111 patients with heart disease of various types were described in detail. This description served as the basis for formulating criteria for the classification of degenerative changes as mild, moderate or severe.

Publications: Ferrans, V.J.: Ultrastructure of degenerated muscle cells in patients with cardiac hypertrophy. To be published as a book chapter in Riecker, G. (Ed.), Myocardial Failure, Munich, West Germany

Ferrans, V.J.: Myocardial ultrastructure in human cardiac hypertrophy. To be published as a book chapter in Myocardiopathies and Myocardial Biopsy, Kaltenbach, M. (Ed.), Frankfurt, Germany, Springer Verlag

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03092-01 PA

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Ultrastructural Aspects of Cardiac Contractile Proteins in 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 — IR PA NHLBI
Other: Barry J. Maron, Senior Investigator, Cardiology Branch — IR CB NHLBI
Michael Jones, Senior Resident, Department of Surgery, Johns Hopkins Hospital
Klaus U. Thiedemann, Guest Worker, Ultrastructure Sec. — IR PA NHLBI

COOPERATING UNITS (if any)

Department of Surgery, Johns Hopkins Hospital, Baltimore, Maryland

LAB/BRANCH
Pathology Branch

Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.2	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)

This project consists of a detailed review of ultrastructural changes involving cardiac contractile proteins in patients with cardiac hypertrophy and degeneration. Emphasis is placed on changes that occur in the late stages of hypertrophy in association with irreversible cardiac failure.

1014

Project No. Z01 HL 03092-01 PA

Project Description: A review was made of morphologic changes in contractile elements of cardiac muscle cells in hypertrophy and failure, with emphasis on alterations occurring in human hearts in the late stages of hypertrophy. The formation of new sarcomeres involves the synthesis of a variety of different proteins, their aggregation into filaments, and the organization of filaments into specific tridimensional arrays. Z-band material appears to play an organizer role in the formation of new sarcomeres. The ultrastructural appearance of contractile elements in hypertrophied muscle cells varies according to the cause and the stage of the hypertrophy. Myofibrillar lysis, with preferential loss of thick myofilaments, occurs in the late stages of hypertrophy in association with degenerative changes, including dissociation of intercellular junctions, proliferation of sarcoplasmic reticulum, cellular atrophy and interstitial fibrosis.

Publications: Ferrans, V.J., Maron, B. J., Jones, Michael, and Thiedemann, K.-U.: Ultrastructural aspects of contractile proteins in cardiac hypertrophy and failure. Proceedings of the 8th International Meeting of the International Study Group for Research in Cardiac Metabolism. Sano, T. and Rona, G. (Eds.), Baltimore, University Park Press.

1015

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03093-01 PA

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Spherical Microparticles in Human Myocardium

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	IR PA NHLBI
Other:	Klaus-Ulrich Thiedemann	Guest Worker, Ultrastructure Sec.	IR PA NHLBI
	Barry J. Maron	Senior Investigator, Cardiology Br.	IR CB NHLBI
	Michael Jones	Senior Resident, Department of Surgery, Johns Hopkins Hospital	
	William C. Roberts	Chief, Pathology Branch	IR PA NHLBI

COOPERATING UNITS (if any)

Cardiology Branch, NHLBI, and Department of Surgery, Johns Hopkins Hospital, Baltimore, Maryland

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	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)

Clusters of spherical microparticles (SMP) that averaged 500 Å in diameter and were composed of dense cores surrounded by single trilaminar membranes were found in operatively obtained biopsies from 29 of 70 patients with various types of heart diseases. SMP appear to form in heart as part of a process that mediates the remodeling of cellular surfaces, especially those of intercellular junctions undergoing dissociation.

1016

Project No. Z01 HL 03093-01 PA

Project Description: Clusters of spherical microparticles (SMP) that averaged 500 Å in diameter and were composed of dense cores surrounded by single trilaminar membranes were found in operatively obtained myocardial biopsies from 29 of 70 patients with various types of heart diseases including: left atrial myocardium (14 patients) and right atrial myocardium (four patients) of 14 patients with mitral valvular disease; left ventricular myocardium of three of 16 patients with aortic valvular disease, three of 16 patients with hypertrophic cardiomyopathy, and two of four patients with combined mitral and aortic valvular disease; and crista supraventricularis muscle of seven of 20 patients with congenital heart diseases associated with muscular obstruction to right ventricular outflow. SMP were consistently associated with interstitial fibrosis and with degeneration of the muscle cells. SMP occurred along the outer surfaces on the sides and free ends of muscle cells in areas of fibrosis, in the widened spaces between membranes of partially dissociated intercellular junctions, and within cytoplasmic vesicles considered to be phagocytic. SMP frequently were joined together by minute nexuses that were structurally identical with those forming parts of intercellular junctions of muscle cells. Evidence is presented to show that SMP occur commonly in tissues other than myocardium. It is concluded that SMP form in the heart as part of a process that mediates the remodeling of cellular surfaces, especially those of intercellular junctions undergoing dissociation.

Publications: Ferrans, V.J., Thiedemann, K-U., Maron, B. J., Jones, Michael, and Roberts, W. C.: Spherical microparticles in human myocardium. An ultrastructural study. Lab Invest 35: 349-368, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03094-01 PA

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Left Atrial Ultrastructure in Mitral Valvular Disease

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 — IR PA NHLBI
Other: Klaus-Ulrich Thiedemann, Guest Worker, Ultrastructure Section — IR PA NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.25	0.10	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)

Light and electron microscopic observations were made on left atrial myocardium of patients undergoing operative repair of mitral valvular disease. Degenerative changes were widespread and severe, and were most prominent in patients having mitral regurgitation and atrial fibrillation.

1018

Project No Z01 HL 03094-01 PA

Project Description: Light and electron microscopic observations were made on left atrial biopsies of 14 patients undergoing operative repair of mitral valvular disease. In fibrotic areas, present in all biopsies, the muscle cells were frequently isolated from each other and exhibited degenerative changes of variable severity. These changes consisted of: lysis of myofibrils, with preferential loss of myosin filaments; proliferation of Z-band material; increased content of cytoskeletal (100 Å diameter) filaments; proliferation of elements of sarcoplasmic reticulum (SR), with formation of aggregates of hexagonally arranged tubules of free SR and of large complexes of cisterns of extended junctional SR; dissociation of intercellular junctions, with formation of spherical microparticles and intracytoplasmic junctions; and accumulation of lysosomal degradation products. These changes were considered to present the end stages of hypertrophy of atrial muscle cells.

Publications: Thiedemann, K.-U., and Ferrans, V.J.: Ultrastructure of left atrial myocardium in patients with mitral valvular disease. To be published as a book chapter in Myocardiopathies and Myocardial Biopsy, Kaltenbach, M. (Ed.), Frankfurt, Germany, Springer Verlag.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03095-01 PA

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Striated Membranous Structures in Human Hearts

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 IR PA NHLBI
Other: Rentería, V. G., Guest Worker, Ultrastructure Section IR PA NHLBI
Jones, Michael, Senior Resident, Department of Surgery
Johns Hopkins Hospital

COOPERATING UNITS (if any)

Department of Surgery, The Johns Hopkins Hospital, Baltimore, Maryland

LAB/BRANCH
Pathology Branch

SI
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.25	0.15	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)

Striated membranous structures which consisted of sheets or ribbons of 130 to 220 Å in thickness and showed variable patterns of periodic substructure were found in extracellular locations in atrioventricular valves, myocardium and endocardium of six patients with various types of heart disease. These structures may result from an unusual pattern of arrangement of basement membrane material.

1020

PHS-6040
(Rev. 10-76)

Project Description: Striated membranous structures (SMS), which consisted of sheets or ribbons of 130 to 220 Å in thickness, showed variable patterns of periodic substructure, and resembled SMS described in renal and ocular tissues in various diseases, were found in extracellular locations in a) mitral valve (2 patients) and tricuspid valve (1 patient) of 2 patients with mitral valvular prolapse, b) mitral valve and femoral artery of 1 patient with Marfan's syndrome and prolapsed mitral valve, and c) myocardium (2 patients) and thickened endocardium (3 patients) of 3 patients with congenital heart disease associated with muscular obstruction to right ventricular outflow. Striated membranous structures measured up to several microns in diameter, often were highly folded and convoluted, and sometimes appeared circular in outline. Some SMS measured from 130 to 150 Å in thickness and had indistinct edges and poorly defined periodicity. The majority of SMS, however, had greater thicknesses, in the range of 200 Å, and a periodicity characterized by alternating light and dark bands with a spacing that varied from 100 to 160 Å. The structures were associated with thickened basement membranes, elastic fibers, and membrane-bound bodies of the type thought to be involved in elastogenesis. Evidence available suggests that SMS result from an unusual pattern of arrangement of a component, possibly Type IV collagen, of basement membrane material.

Publications: Rentería, V.G., Ferrans, V.J., and Jones, M.: Striated membranous structures in human hearts. An ultrastructural study. Am J Pathol 85: 85-98, 1976

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03096-01 PA

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Extended Junctional Sarcoplasmic Reticulum in Human Left Atrial Myocardium

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 IR PA NHLBI
Other: Klaus-Ulrich Thiedemann, Guest Worker, Ultrastructure Section IR PA NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Pathology Branch

SL
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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)

Extended junctional sarcoplasmic reticulum was observed in left atrial muscle cells from 11 patients with mitral valvular disease, and was considered to represent an extreme form of overdevelopment of sarcoplasmic reticulum in response to the stimulus of hypertrophy.

1022

PHS-6040
(Rev. 10-76)

Project No. Z01 HL 03096-01 PA

Project Description: Extended junctional sarcoplasmic reticulum is defined as having an electron-dense content, similar to that of junctional sarcoplasmic reticulum (i.e., junctional granules), but not establishing specialized contacts with the sarcolemma. This report, the first description of extended junctional sarcoplasmic reticulum in human myocardium, describes its occurrence in left atrial myocardium from 11 patients with mitral valvular disease. It is concluded that the formation of large amounts of extended junctional sarcoplasmic reticulum is an extreme form of overdevelopment of sarcoplasmic reticulum of atrial muscle in response to the stimulus of hypertrophy.

Publications: Thiedemann, K.T., and Ferrans, V.J.: Occurrence of extended junctional sarcoplasmic reticulum in atrial myocardium of patients with mitral valvular disease. In Ben-Shaul, Y. (Ed.): Electron Microscopy 1976. Proceedings of the 6th European Congress on Electron Microscopy, Jerusalem, Israel, September 14-20, 1976. Israel, Jerusalem, Tal International Publishing Co., 1976, Vol. II, Biological Sciences, pp. 433-435.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03097-01 PA

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Myocardial Degeneration in Congenital Heart Disease

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 IR PA NHLBI
Other: Michael Jones, Department of Surgery, Johns Hopkins Hospital

COOPERATING UNITS (if any)
Department of Surgery, Johns Hopkins Hospital

LAB/BRANCH
Pathology Branch

S.
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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)

Electron microscopic study of operatively removed myocardium from patients with congenital heart diseases associated with muscular obstruction to right ventricular outflow showed that degenerative changes in cardiac muscle cells were much more prevalent and severe in a group of 8 patients aged 30 to 53 years than in a group of 11 patients aged 1 to 5 years.

1024

PHS-6040
(Rev. 10-76)

Project Description: A comparison was made of electron microscopic findings in operatively resected crista supraventricularis muscle from 11 patients aged 1 to 5 years (Group 1) and from 8 patients aged 30 to 53 years (Group 2) with congenital heart disease associated with muscular obstruction to right ventricular outflow. Of the 11 patients in Group 1, none had cardiac failure or arrhythmias preoperatively; 2 patients died after operation, both of consequences of unrelieved right ventricular hypertension, and the others did well postoperatively. In contrast, five of the eight patients in Group 2 had right ventricular failure preoperatively; three had clinically significant arrhythmias and three died after operation (two of low cardiac output and one of arrhythmias). Changes of hypertrophy (cells over 20 μ in diameter, lobulated nuclei, multiple intercalated discs, dilated T tubules and increased numbers of ribosomes) were present in both groups but were more prominent in the older patients. Marked interstitial fibrosis, cellular atrophy, myofibrillar disorganization, myelin figures, myofibrillar lysis, proliferation of smooth endoplasmic reticulum, lipid deposition, spherical microparticles associated with the plasma membranes of the muscle cells, intracytoplasmic junctions and thickening of the basal laminae of the muscle cells were entirely absent in the younger patients, although common in the patients over 30 years old. Other changes common in Group 2 (cellular disorganization, intramitochondrial glycogen and abnormal Z bands) were infrequent in Group 1. Ultrastructural changes of severe degeneration in the patients over 30 years old appeared to reflect the stresses of prolonged right ventricular hypertrophy and hypoxia and correlated with clinical cardiac dysfunction.

Publications: Jones, M. and Ferrans, V.J.: Myocardial degeneration in congenital heart disease. Comparison of morphologic findings in young and old patients with congenital heart disease associated with muscular obstruction to right ventricular outflow. Am J Cardiol 39: 1051-1063, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03098-01 PA

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Fiber Lesions in Cardiomyopathy of Selenium-vitamin E Deficient Swine

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 IR PA NHLBI
Other: John F. Van Vleet, Purdue University School of Veterinary Medicine
George Ruth, Purdue University School of Veterinary Medicine

COOPERATING UNITS (if any)

Purdue University School of Veterinary Medicine, Lafayette, Indiana

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.20	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)

An ultrastructural study was made of the lesions that developed in cardiac muscle cells of pigs fed a semisynthetic diet deficient in selenium and vitamin E. These lesions had features of myofibrillar degeneration, with hypercontraction bands, myofibrillar lysis and mitochondrial damage.

1026

PHS-6040
(Rev. 10-76)

Project Description: Cardiomyopathy was produced in 38 weanling swine by feeding them a semisynthetic diet deficient in selenium and vitamin E (Se-E) for 13 to 59 days. Pigs were killed for morphologic studies of the cardiac lesions at sequential times after development of the deficiency disease. Gross examination disclosed hydropericardium and scattered pale streaks and patches of necrosis in the myocardium, especially the left ventricle. Histopathologically, the lesions were scattered throughout the heart but were most severe in the atria. Ultrastructurally, the damaged fibers had many features of myofibrillar degeneration, with hypercontraction bands, myofibrillar lysis, and mitochondrial swelling, disruption and mineralization. Numerous macrophages appeared to have passed through focal disruptions in the external laminae of the muscle cells and engulfed sarcoplasmic and nuclear debris. Stromal collapse and mild fibrosis persisted as residual lesions in scattered areas of myocardium in pigs with long-term deficiency. Although vascular lesions were present in the hearts of Se-E deficient pigs, it was concluded that the fiber alterations developed independently of the vascular changes. The pathogenesis of this cardiomyopathy induced by nutritional deficiency is thought to be related to lack of protection by the seleno-enzyme, glutathione peroxidase, and the antioxidant, vitamin E, from lipoperoxidative damage.

Publications: Van Vleet, J.F., Ferrans, V.J., and Ruth, G.R.: Ultrastructural alterations in nutritional cardiomyopathy of selenium-vitamin E deficient swine. I. Fiber lesions. Lab Invest (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03099-01 PA

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Cardiac Lesions Induced by Multiple Defibrillator Shocks

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 IR PA NHLBI
Other: John F. Van Vleet, Purdue University School of Veterinary Medicine
W. A. Tacker, Purdue University Biomedical Engineering Center
L. A. Geddes, Purdue University Biomedical Engineering Center

COOPERATING UNITS (if any)

Purdue University School of Veterinary Medicine, and Purdue University Biomedical Engineering Center

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.20	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)

An ultrastructural study was made of cardiac lesions produced in dogs by multiple transthoracic shocks of various strengths delivered by a trapezoidal waveform defibrillator. These lesions had features of myofibrillar degeneration and were related to the strength of the current used.

1028

PHS-6040
(Rev. 10-76)

Project Description: Eighteen normal dogs were given single or multiple transthoracic shocks with a trapezoidal waveform defibrillator. Of the 7 dogs that received 6 suprathreshold shocks (3.2 to 10.6 amps/kg), 1 died within 20 minutes and 4 had myocardial lesions when killed 2 hours after shocking. The remaining 11 dogs, which were given fewer shocks and/or less current dose per shock, (1.2 to 1.9 amp/kg) did not have cardiac damage. Electrocardiographic changes occurred more often in dogs with cardiac damage than in dogs without cardiac damage. Lesions in dogs with cardiac damage were apparent grossly as discrete, pale, somewhat turgid areas in the lateral walls of both ventricles, and were consistently located in a path between the externally placed electrodes. Microscopic and ultrastructural study of damaged areas revealed cardiac muscle cells with myofibrillar degeneration. In mildly injured cells, which probably were reversibly damaged, the ultrastructural alterations included: 1) damage to the plasma membrane and to membranes of mitochondria and sarcoplasmic reticulum, and 2) intracellular edema, characterized by prominent subsarcolemmal and paranuclear vacuoles. Severely injured cardiac muscle cells showed increased membrane damage, strikingly prominent myofibrillar hypercontraction bands and nuclear pyknosis. The surrounding connective tissues were edematous. Cardiac muscle cell damage appeared to evolve from initial membrane injury, with resultant loss of cellular volume control and development of disturbed intracellular electrolyte balance, leading to subsequent myofibrillar and nuclear alterations. It is concluded that the use of the trapezoidal waveform defibrillator is attended by a safety margin between the doses of electrical current required for effective defibrillation and for inducing cardiac damage, but that sizeable overdoses can lead to cardiac damage and even to immediate post-shock mortality as has been described with other types of defibrillators.

Publications: Van Vleet, J.F., Tacker, W. A., Jr., Geddes, L. A., and Ferrans, V.J.: Acute cardiac damage in dogs given multiple transthoracic shocks with a trapezoidal wave defibrillator. Am J Vet Res (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03100-01 PA

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Intracellular Collagen Fibrils in Prolapsed Human Atrioventricular 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 IR PA NHLBI
Other: Vicente G. Rentería, Guest Worker, Ultrastructure Sec. IR PA NHLBI
Michael Jones, Senior Resident, Department of Surgery, Johns Hopkins Hospital
William C. Roberts, Chief, Pathology Branch IR PA NHLBI

COOPERATING UNITS (if any)
Department of Surgery, Johns Hopkins Hospital, Baltimore, Maryland

LAB/BRANCH
Pathology

SL
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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)

Intracellular collagen fibrils were present in granular cells in atrioventricular valves of 5 patients with the syndrome of atrioventricular valve prolapse. These fibrils may form as a consequence of abnormal interactions between newly secreted collagen and acid mucopolysaccharides.

1030

PHS-6040
(Rev. 10-76)

Project No. Z01 HL 03100-01 PA

Project Description: Ultrastructural study of prolapsed atrioventricular valves from five patients, one of whom also had the Marfan syndrome, disclosed granular cells that were characterized by numerous membrane-bound, electron-dense inclusions resembling those in the Hurler syndrome. Collagen fibrils were present within some of these inclusions. The intracellular collagen deposits may form as a consequence of abnormal interactions between newly secreted collagen and acid mucopolysaccharides.

Publications: Rentería, V. G., Ferrans, V. J., Jones, Michael, and Roberts, W. C.: Intracellular collagen fibrils in prolapsed ("floppy") human atrioventricular valves. Lab Invest 35: 439-443, 1976

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03101-01 PA

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Vascular Lesions in Cardiomyopathy of Selenium-vitamin E Deficient Swine

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 IR PA NHLBI
Other: John F. Van Vleet, Purdue University School of Veterinary Medicine
George R. Ruth, Purdue University School of Veterinary Medicine

COOPERATING UNITS (if any)

Purdue University School of Veterinary Medicine, Lafayette, Indiana

LAB/BRANCH
Pathology Branch

Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.25	0.15	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)

An ultrastructural study was made of the myocardial arteriolar lesions that developed in pigs fed a semisynthetic diet deficient in selenium and vitamin E. These lesions were characterized by the presence of fibrinoid material in the vascular walls.

1032

PHS-6040
(Rev. 10-76)

Project No. Z01 HL 03101-01 PA

Project Description: Selenium-vitamin E (Se-E) deficiency was produced in weanling swine by feeding a semisynthetic basal diet for 13 to 59 days. Pigs were killed sequentially for morphologic studies of the cardiac lesions. In hearts with vascular damage, gross hemorrhages were scattered in the myocardium and serosal surfaces. Light and electron microscopic study revealed myocardial arteriolar damage characterized by segmental fibrinoid accumulation in vessel walls and by scattered fibrin thrombi. Ultrastructural study disclosed extensive subendothelial and inner wall accumulations of dense granular deposits of serum proteins and masses of fibrin in arterioles in which dense deposits of fibrinoid were identified by light microscopy. Endothelial cells of these arterioles were loosely attached to each other. In arterioles with fibrin thrombi, the endothelium was disrupted. In mildly injured arterioles, increased endothelial permeability resulted in insudation of blood proteins into the vessel wall to produce accumulation of fibrinoid. In severely injured vessels, endothelial integrity was destroyed, smooth muscle cells were necrotic and thrombosis had developed. Initiation of these arteriolar lesions was apparently the result of lipoperoxidative damage to endothelial cell membranes that lacked protection by Se-E.

Publications: Van Vleet, J.F., Ferrans, V.J., and Ruth, G.R.: Ultrastructural alterations in nutritional cardiomyopathy of selenium-vitamin E deficient swine. II. Vascular lesions. Lab Invest (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03102-01 PA

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Pathology of Rheumatic Heart Disease

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 — IR PA NHLBI
Other: William C. Roberts, Chief, Pathology Branch — IR PA NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Pathology Branch

S:
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.15	0.10	0.05

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)

This project consists of a detailed review of cardiac pathologic changes produced by acute rheumatic fever and by chronic rheumatic heart disease.

1034

Project Description: This project describes the pathology of acute rheumatic fever and of chronic rheumatic heart disease. Aschoff nodules, the characteristic feature of acute rheumatic fever, are spindle-shaped collections of cells and altered connective tissue. They follow a cycle of development and resolution which begins with an exudative-degenerative phase, continues to a stage of maturity (granulomatous phase) and eventually concludes with healing by fibrosis. Aschoff nodules contain: Aschoff cells; Anitschkow cells; plasma cells; lymphocytes; fibrinoid material; focally swollen, hypereosinophilic and fragmented bundles of collagen, and, in the early stages of the lesions, neutrophilic and polymorphonuclear leucocytes. As they continue to evolve, fibrinoid material disappears and the lesions become progressively more fibrotic. Ultrastructural features of all these components are described.

The chronic valvular lesions caused by the rheumatic process also are described. Emphasis is placed on the following facts: 1) that valvular disease limited to the aortic valve is nonrheumatic in origin; 2) that rheumatic fever practically always involves the mitral valve, and 3) that nonrheumatic disease of the mitral valve is much more common than is generally appreciated.

Publications: Ferrans, V.J., and Roberts, W. C.: Pathology of rheumatic heart disease. To be published as a book chapter in Special Aspects of the Surgery of Rheumatic Heart Disease in Children, Gotsman, M. and Borman, J.B. (Eds.)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03103-01 PA

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Cardiomyopathy Induced by Antineoplastic Drugs

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 IR PA NHLBI
Other: Eugene H. Herman, Division of Drug Biology, Food and Drug Administration

COOPERATING UNITS (if any)

Division of Drug Biology, Food and Drug Administration

LAB/BRANCH
Pathology Branch

SI
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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)

Two types of cardiomyopathy are induced by drugs used in the treatment of cancer. The first type is a chronic, dilated-type caused by anthracyclines, the second is an acute type caused by high-dose combination therapy with cyclophosphamide.

1036

PHS-6040
(Rev. 10-76)

Project No. Z01 HL 03103-01 PA

Project Description: Two types of cardiomyopathy are induced by drugs used in the treatment of cancer. The first type, produced by anthracycline drugs (adriamycin and daunorubicin), is characterized by congestive heart failure, cardiac dilatation and structural evidence of myocardial cellular degeneration. The second type, which results when high doses of cyclophosphamide are used in combination with other chemotherapeutic agents to ablate bone marrow in preparation for bone marrow transplantation, is manifested by acute, fulminating cardiac failure, pericarditis and myocardial microthrombosis.

Publications: Ferrans, V.J., and Herman, E.H.: Cardiomyopathy induced by antineoplastic drugs. In Kaltenbach, M. (Ed.), Myocardiopathies and Myocardial Biopsy. Frankfurt, Germany, Springer Verlag (in press)

ANNUAL REPORT OF THE
SECTION ON THEORETICAL BIOPHYSICS
OFFICE OF THE DIRECTOR OF INTRAMURAL RESEARCH
NATIONAL HEART, LUNG, & BLOOD INSTITUTE
July 1, 1976 through September 30, 1977

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.

During the past year, areas of work have included: (1) The thermodynamics analysis of flow processes, (2) the qualitative analysis of equations describing kidney models, (3) the development of analytical solution of kidney models, (4) the development and theoretical analysis of numerical methods, (5) the computer simulation of renal function, and (6) the computer simulation of solute and water transport in simple epithelia.

Specifically (1) Earlier work on the contribution of viscous dissipation to entropy production in a network of flow tubes has been written up in final form and has been accepted for publication. In this work it was shown that viscous dissipation contributes terms of the form $\int_0^{L_i} R_{iF}(F_{iv})^2 dx$ to entropy production, where L_i is the length, R_{iF} the flow resistance, and F_{iv} the volume flow in a given renal tubule or capillary. The theory has been applied to the calculation of entropy production in a simple model of the medulla.

(2) A major effort has been expanded on the qualitative analysis of the differential equations describing kidney models. The general purpose of this work is to establish conditions (e.g., parameter ranges in the phenomenological equations governing transmembrane transport) that guarantee the existence and uniqueness of solutions of the differential equations describing kidney models. This work has been motivated in part by the discovery that for certain simple models there are parameter ranges for which solutions either do not exist or are non-unique. Empirically, in numerical calculations on more sophisticated models non-unique solutions are found. A question of real physiological interest is whether some of the mathematical pathology, such as flow reversal in collecting duct, can occur as real pathology in the kidney under suitable conditions. A related question is the extent to which regulation of the kidney is determined by membrane parameters and the extent to which it depends on intrinsic or extrinsic feedback mechanisms. This work has been carried out in collaboration with Prof. Bruce Kellogg, University of Maryland, and Prof. Jackie Garner, Louisiana Tech University, Ruston, Louisiana. It has been supported in part by Contract # HI52900.

(3) An extension of earlier work on the analytical theory of kidney models has led to a precise relation between the classic clearance concept of negative free water, $-T^c_{H_2O}$, and the total net solute transport out of the ascending limb of Henle, $T_{s,AHL}$. The negative free water, by definition, is the volume of water that would have to be added to a hypertonic urine to return it to plasma osmolality. Of the solute supplied by the ascending limb system, a fraction, $1 - f_W$, where f_W is a measure of the dissipation of the counterflow system by the vasa recta, is used to extract water from the loop of Henle and the collecting duct. Of this total, a fraction f_U is used to extract water from the collecting duct, where f_U is the ratio of final urine flow to combined flow in collecting duct and descending Henle's limb. Combining these factors leads to the final expression for negative free water $-T^c_{H_2O} = (1 - f_W)\, f_U\, T_{s,AHL}$. Thus, a classic clearance concept is related to the analytic theory of medullary counterflow systems.

(4) Several different projects have been completed on the development and analysis of numerical methods for solving the kidney equations. In general this work is directed toward faster, more efficient, and more accurate solutions of the equations. a) Analytic computation of the Jacobian matrix used for solving individual tube equations has been incorporated into the multinephron model of the kidney. This has resulted in a substantial savings in the time (and cost) of running this model.

b) Following up on numerical experiments done by Tewarson (unpublished), we have included volume flows in addition to concentration and pressure in the vector of unknowns (previous volume flows were eliminated by integration). This has increased the radius of convergence. As a result, an initial estimate can be further from the final solution, and the final solution can be obtained in fewer iterations.

c) A polynomial approximation method for solving the kidney models has been tested in a 4-tube model of the medulla that includes ascending and descending vasa recta, descending limb of Henle, and interstitium. When compared with the conventional trapezoidal scheme we have used in most of our calculations, the polynomial approximation method saved much storage space, some cpu time, and decreased the oscillations in the transmural fluxes for comparable truncation error. The method appears promising for more detailed models of the kidney.

d) A study has been made of some numerical methods for the solution of two point boundary value problems. The motivation for this came from the thought that the key to reducing the memory and running time requirements for multinephron models lies in solving the interstitial equations more accurately. Specifically, an attempt was made to find schemes that guarantee a positive solution as diffusion terms become negligible relative to bulk

flow terms. This has been accomplished with first and second order schemes. For these schemes a detailed error analysis has been developed (In collaboration with B. Kellogg).

e) Various higher order integration schemes were tested. These included cubic and fifth degree overhang and a corrected trapezoidal rule. These gave a smaller truncation error than the conventional trapezoidal integration schemes for smooth data, but required special treatment in the vicinity of discontinuities such as the transition from the inner to the outer medulla. In order to make the schemes more efficient, sparse matrix routines are being incorporated into them (with Tewarson - supported in part by Contract 201 HL 030202).

(5) Simulation of renal function has been extended with the multinephron model of the whole kidney and the multinephron central core model of the renal medulla. With these models, a detailed study has been initiated on the role of urea cycling in the concentration of urine. This study has confirmed the previous finding that a substantial concentration gradient can be generated in the inner medulla with no active salt transport out of thin ascending limb of Henle. In the development of this gradient, the ratio of cortical to juxtamedullary nephrons is a very critical parameter. On the basis of our calculations so far, we find a ratio of 9 cortical to 1 juxtamedullary is about optimum.

Work has also begun on modifying the multinephron model so as to be able to simulate input/output curves of radioactive tracer. There is considerable current interest in the use of such washout data to evaluate the viability of kidney transplants. We hope that this work will lead to improved diagnostic methods in this area.

(6) Water transport in a single solute model of a leaky epithelia (e.g. proximal tubule) that includes both cellular and shunt pathways has been studied. It has been found that in many respects the steady state performance of the system is not affected by the cellular compartment. This probably reflects the fact that in a single solute model the problem of osmotic regulation of the cellular compartment is in part finessed. Work has begun on extending this model to include transient behavior and addtional solutes.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03201-17 STB

PERIOD COVERED

July 1, 1976 through September 30, 1977

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		
	K. Crump	Prof. Louisiana Tech University, Ruston, La.		
	J. Garner	Prof. Louisiana Tech University, Ruston, La.		
	A. Weinstein	Research Associate		

COOPERATING UNITS (if any)

Mathematical Research Branch, NIAMDD: IPST, University of Maryland, Louisiana Tech University, Ruston, La.

LAB/BRANCH

SECTION

Section on Theoretical Biophysics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.8	.6	.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 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 analysis of flow processes, (2) the qualitative analysis of equations describing kidney models, and (3) the development of analytical solutions of kidney models.

1043

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 analysis of flow processes; the qualitative analysis of equations describing kidney models, and the development of analytical solutions of kidney models. Each topic will be considered in turn.

Specific Findings: (1) Earlier work on the thermodynamic analysis of flow process with viscous dissipation was written up in final form and has been accepted for publication. No further theoretical extension of this work is planned for the present. It has now been brought to the point where the calculation of various dissipative effects can be incorporated into our models (with A. Weinstein).

(2) Work on the qualitative theory of the differential equations describing kidney models has continued as a major effort. The primary goal of this research is to establish existence and uniqueness theory for the steady state boundary value problems that model the renal counterflow systems. Such results are desirable in that they would define parameter ranges and flux formulas that guarantee the mathematical validity of the models. Under these conditions, investigators could place more confidence in their numerical studies of both steady state problems and transient problems. This work is partly motivated by the fact that some relatively simple models have either no solution or solutions that are non-unique for certain choices of parameters (Stephenson and Kellogg - unpublished).

The counterflow system considered in this work consists of n parallel flow tubes, each of length L, in each of which a fluid-solute mixture is flowing. These tubes may interchange fluid or solute with one another by an interchange through the tube walls. So far we have considered systems with one solute and a constant pressure.

To derive the differential equations for the system we let $C_i(x)$ and $F_i(x)$ denote the steady state solute concentration and volume flow, respectively, in the ith tube. We denote by $J_{sij}(x,C_i,C_j)$ and $J_{vij}(x,C_i,C_j)$ respectively, the trans-membrane solute flux and volume flux from tube i to tube j. Then

$$J_{si} = \sum_{j=1}^{n} J_{sij} \text{ and } J_{vi} = \sum_{j=1}^{n} J_{vij}$$

denote, respectively, the total trans-membrane solute flux and volume flux out of tube i. Letting $D_i \geq 0$ be the diffusion coefficient of the solute in tube i, we find the system of differential equations for the $C_i(x)$, $F_i(x)$, $i = 1, \ldots, n$, to be

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$$- D_i C_i'' + (F_i C_i)' = - J_{si}(x, C_1, \ldots, C_n)$$

$$F_i' = - J_{vi}(x, C_1, \ldots, C_n)$$

Along with the system of differential equations we impose the boundary conditions as defined in the following description of the system of tubes.

At $x = 0$ certain pairs of tubes will be considered as attached to each other. The remaining tubes will be either open or closed at $x = 0$. A similar condition will hold at $x = L$. If tube i and tube j are attached at $x = 0$ or $x = L$, the boundary conditions there are

$$C_i = C_j, \quad D_i C_i' = - D_j C_j', \quad F_i = - F_j.$$

If tube i is open at $x = 0$ or $x = L$ the value of the concentration there is a specified positive number,

$$C_i(0) = C_{i0} > 0 \quad \text{or} \quad C_i(L) = C_{iL} > 0.$$

If tube i is closed at one end, the boundary conditions there are

$$C_i' = 0, \quad F_i = 0.$$

By following a tube i from its open end through perhaps a series of attached tubes, attached by pairs at the boundaries $x = 0$ and $x = L$, one reaches a tube, say k, with either a closed end (and $C_k' = 0$ and $F_k = 0$ are imposed) or an open end. In the latter case the boundary condition is either a specified value for F_i at the open end of tube i or for F_k at the open end of tube k (but not both) along with the specified concentrations at the open ends of the two tubes.

The problem then is to determine general conditions for the $J_{si}(x, C_1, \ldots, C_n)$, $J_{vi}(x, C_1, \ldots, C_n)$ so that these boundary value problems will have unique solutions for the C_i, F_i. In an effort to establish this existence and uniqueness theory, we have obtained a number of results for the cases $n = 1$ and $n = 2$. Also, we have obtained an existence result for the n-tube problem.

The results were obtained under the hypotheses

H_1. $J_{si}(x, C_1, \ldots, C_{i-1}, 0, C_{i+1}, \ldots, C_n) < 0$

when each $C_j \geq 0$, $j = 1, 2, \ldots, n$,

and

H_2. there exists a constant M,

$M > \max$ {given concentrations at $x = 0$ and $x = L$}

such that for each i

$$J_{si}(x,C_1, \ldots, C_n) - C_i J_{vi}(x,C_1, \ldots, C_n) > 0$$

when $C_i \geq M$ and $C_i \geq C_j \geq 0$, $j = 1, \ldots, n$.

A brief summary of these results is now given.

a. $n = 1$

The differential equations, for $n = 1$, have been studied with four different sets of boundary conditions. For $D_1 > 0$ the conditions are either

$$C_1'(0) = 0, \quad C_1(L) = C_L, \quad F_1(0) = 0$$

or

$$C_1(0) = C_0 > 0, \; C_1(L) = C_L > 0, \; F_1(0) = F_0.$$

For $D_1 = 0$ the conditions are

$$C_1(0) = C_0 > 0, \; F_1(0) = F_0 > 0$$

or

$$C_1(0) = C_0 > 0, \; F_1(L) = F_L > 0.$$

In this one tube model, an exchange of fluid and solute in the tube with that in the interstitium that contains the tube is allowed. The interstitium concentration is considered as a known function of x.

The existence of solutions of these four problems was established under the hypotheses H_1, H_2, and that each $J_{si}(x,C_1)$, $J_{v1}(x,C_1)$ is continuous on $[0,L] \times [0,M]$. For the case $D_1 = 0$, an additional condition requiring F_0 and F_L to be sufficiently large,

$$F_0 - \int_0^x J_{v1}(u,C_1(u))\, du \geq \alpha > 0 \quad \text{on } [0,L]$$

$$F_L + \int_x^L J_{v1}(u,C_1(u))\, du \geq \alpha > 0 \quad \text{on } [0,L]$$

was imposed. The uniqueness of solutions for these four problems was then obtained by adding to the hypotheses

H_3. $-J_{s1}(x,C_1)$ and $J_{v1}(x,C_1)$ are nonincreasing in C_1, with one strictly monotonic, on $[0,L] \times [0,M]$.

1046

It was then shown that these hypotheses are satisfied, and thus our results apply, when J_{s1} and J_{v1} are taken by the typical formulas

$$J_{s1} = h_1(C_1 - C_I(x)) + \frac{a\,C_1}{b + C_1} - \frac{a_I C_I(x)}{b_I + C_I(x)} + \frac{\sigma h_2}{2}(1 - \sigma)(C_I^2(x) - C_1^2)$$

$$J_{v1} = \sigma h_2 (C_I(x) - C_1), \quad 0 \le \sigma \le 1,$$

provided certain inequalities are satisfied by the nonnegative coefficients

$$h_1, h_2, \sigma, a, b, a_I, b_I.$$

b. $n = 2$

Similar results have been obtained for the case $n = 2$. Here the boundary conditions were taken as

$$C_1(0) = C_{10} > 0 \qquad F_1(0) = F_0$$

$$C_2(0) = C_{20} > 0$$

$$C_1(L) = C_2(L) \qquad F_1(L) = - F_2(L)$$

$$C_1'(L) = - C_2'(L)$$

when $D_1 D_2 > 0$ and

$$C_1(0) = C_0 > 0 \qquad F_1(0) = F_0 > 0$$

$$C_1(L) = C_2(L) \qquad F_1(L) = - F_2(L)$$

when $D_1 = D_2 = 0$. Such conditions depict a system of two flow tubes which are connected at the end $x = L$.

For $D_1 D_2 > 0$ the existence of a solution of the boundary value problem was obtained under the hypotheses H_1, H_2 (for $n = 2$) and that each

$J_{si}(x, C_1, C_2)$, $J_{vi}(x, C_1, C_2)$ is continuous for $0 \le x \le L$, $0 \le C_j \le M$, j=1,2.

For $D_1 = D_2 = 0$, our results require that the J_{si}, J_{vi} have the form

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$$J_{s1} = J_s(x, C_1, C_2) + h_1(x, C_1, C_2) - g_1(x)$$

$$J_{s2} = -J_s(x, C_1, C_2) + h_2(x, C_1, C_2) - g_2(x)$$

$$J_{v1} = -J_{v2} = h_v(C_2 - C_1)$$

where $g_i(x) \geq 0$ and $h_i(x, C_1, C_2) \geq 0$ when $C_1, C_2 \geq 0$, $i = 1,2$. The existence result was obtained under the hypotheses H_1, H_2, and $F_0^2 > f(L)$ where

$$f(x) = 2h_v \int_0^x \int_u^L [g_1(r) + g_2(r)]\, dr du.$$

Uniqueness of solutions was obtained for the case $h_1(x, C_1, C_2) \equiv h_2(x, C_1, C_2) \equiv 0$. Also, in this case, it was shown that the problem does not have a solution if $F_0^2 \leq f(L)$.

c. *n* arbitrary

Under the hypotheses H_1 and H_2, the existence of a solution to the n-tube problem was established.

d. Transient problem

The transient form of the n tube equations, augmented to include a non-constant pressure, has also been considered. By using a form of the method of lines with a backward implicit time step procedure ("Rothe's method"), the analysis of this problem is reduced to that of an auxiliary steady state problem. Existence and uniqueness of the transient solution is obtained with fairly general hypotheses on the transmembrane flux formulas.

The basic tools used in obtaining the results in this research were the application of a topological fixed point theorem to obtain the existence results and the application of a comparison theorem to obtain the uniqueness results. This work is being written up for publication.

Our plan for future research includes a study of the n tube problem and a study of the effects of pressure and more than one solute on the analysis (Kellogg, B., Contract # HI52900, and Garner, J., Visiting Scientist).

3. The Development of Analytical Solutions of Kidney models

Earlier work on the analytic and semianalytic solution of transient problems has been submitted and accepted for publication (with Crump and Garner).

An extension of earlier work on the analytical theory of kidney models has led to a precise relation between the classic clearance concept of negative free water, $-T^{c}_{H_2O}$ and the total net solute transport out of the ascending limb of Henle, $T_{s,AHL}$. The negative free water, by definition, is the volume of water that would have to be added to a hypertonic urine to return it to plasma osmolality. Of the solute supplied by the ascending limb system, a fraction, $1 - f_W$, where f_W is a measure of the dissipation of the counter-flow system by the vasa recta is used to extract water from the loop of Henle and the collecting duct. Of this total, a fraction f_U is used to extract water from the collecting duct, where f_U is the ratio of final urine flow to combined flow in collecting duct and descending Henle's limb. Combining these factors to the final expression for negative free water $- T^{c}_{H_2O} = (1 - f_W)\, f_U\, T_{s,AHL}$. Thus, a classic clearance concept is related to the analytic theory of medullary counterflow systems.

Proposed Course and Significance to Biomedical Research:

We plan to continue work on the general qualitative analysis of the kidney equations. Eventually this should lead to both better computational methods and greater intuitive insight into our models.

Publications:

*Kellogg, B., Uniqueness in the Schauder Fixed point theorem. Proc. American Math. Soc. 60:207-210, 1976.

*Kellogg, B. A Priori bounds for renal network flows. Conference Proceedings, 1976 Summer Computer Simulation Conference, Washington D. C. pp. 456-460.

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Stephenson, J. L. Analysis of the transient behavior of kidney models. Bull. Math. Biology (In press).

Garner, J. B., Crump, K. S., and Stephenson, J. L. Transient behavior of the single loop solute cycling model of the renal medulla. Bull. Math. Biol. (In press).

Stephenson, J. L., Report on mathematical models of the kidney and its subsystems, to the subcommittee on normal kidney structure and function, NIAMDD Nephrology, Urology Research Needs Survey (In press).

Burg, M. B., Stephenson, J. L., Transport characteristics of the loop of Henle, Chapter 37, part 3, Physiological Basis for Disorders of Biological Membranes, Edited by T. E. Andreoli, J. F. Hoffman, D. Fanestil, Plenum Press (In press).

*Supported by NHLBI under Contract HI 52900

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03202-06 STB

PERIOD COVERED

July 1, 1976 through September 30, 1977

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:	J. L. Stephenson	Chief, Section on Theoretical Biophysics	OD	NHLBI
OTHERS:	R. Mejia	Mathematician	OD	NHLBI
	A. Tsan	Visiting Fellow	OD	NHLBI
	B. Hubbard	Prof. IPST, Univ. of Maryland		
	R. Tewarson	Prof. SUNY, Stony Brook, L. I. New York		
	B. Kellogg	Prof. IPST, Univ. of Maryland		
	R. Huss	Postdoctoral Fellow		

COOPERATING UNITS (if any)

IPDAM, University of Maryland: Dept. of Applied Mathematics and Statistics, SUNY, Stony Brook, L. I. New York, and NIAMDD, Office of Mathematical Research.

LAB/BRANCH

SECTION

Section on Theoretical Biophysics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20014

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 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.

1051

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. In support of the general purpose, work on the development and theoretical analysis of numerical methods is carried out.

Major Findings: 1. The multinephron model of the kidney has both been extended and used for numerical simulations.

(a) The model was modified to compute tubal Jacobians analytically. Solutions of this model have been obtained by solving individual tube stepwise against assumed interstitial concentrations and pressure. This leads to the solution of a large number of nonlinear systems of relatively low order (N system of order $k + 2$ per tube, where N is the number of tubal segments and k is the number of solutes). Since the correction to the solution at each Newton iteration is

$$\Delta\gamma = J^{-1}\phi$$

where ϕ is the vector of residuals and $J^{-1} = (\frac{\partial\phi}{\partial\gamma})^{-1}$ is the inverse of the Jacobian matrix, an analytic expression for J^{-1} is desirable for efficient calculation. For $k > 1$ this is in general not a tractable problem, but an analytic expression for the Jacobian has been incorporated into the model. Although its inversion is done numerically, a substantial saving in computer time results.

(b) For a nonlinear system of order 3 or less, an analytic inverse of the Jacobian has been written. Such a scheme has been incorporated into the multinephron central core medullary model.

(c) Numerical experiments done by Tewarson (unpublished) suggested that inclusion of the volume flow into the vector of unknowns in the solution of the kidney equations would increase the rate of convergence substantially. Up to this time the volume flows had been obtained by integrating numerically the equation

$\frac{dF_{iv}}{dx} = -J_{iv}$ for the ith tube. This had been done in order to reduce the number of simultaneous nonlinear equations to be solved and thus readily permit a finite difference resolution of 0.1 in space (10 space chops per tube).

The flow equations were added to the multinephron multisolute model both in the tubal and interstitial calculations. This addition, in the interstitium in particular, have resulted in a marked stabilization of

the solution. A corresponding increase in the radius of attraction of a solution has resulted. This is especially significant in the transition from one steady state solution to another, such as in going from diuresis to antidiuresis. Formerly, step changes in hydraulic permeabilities might cause flow reversals and subsequent convergence to negative, nonphysiologic solutions. The present model is much less sensitive to such changes.

(d) A detailed analysis of the effects of urea upon the concentrating mechanism has been undertaken. Use is being made of both the central core multisolute multinephron model and of the whole kidney model. The central core model has not only provided good initial data for the whole kidney model but results qualitatively consistent with the more complex and time consuming whole kidney model.

(e) Work has begun on modifying the multinephron model so as to be able to simulate input/output curves of radioactive tracer. There is considerable current interest in the use of washout data to evaluate the viability of kidney transplants. We hope that this work will lead to improved methods for the analysis of washout data.

2. Considerable work has been carried out in the development and analysis of numerical methods.

(a) A study has been made of some numerical methods for the solution of two point boundary value problems. The motivation for this comes from the thought that the key to reducing the memory and running time requirements for the multinephron programs lies in solving the interstitial equations more accurately. The interstitial equations are a system of diffusion-convection equations, and therefore we have studied new ways to solve such equations.

We consider the equation

$$(1) \quad -Du'' + pu' + qu = f,$$

$$p > 0,\ q > 0,$$

$$u(0) = \alpha,\ u(1) = \beta.$$

It is known that when h/D is large, where h is the mesh spacing, the centered three point difference scheme for (1) gives inaccurate results. Our approach to avoiding this is to search for difference schemes that have the property that as $D \to 0$, the difference solution converges to a difference approximation to the reduced problem

$$(2) \quad pu' + qu = f \quad u(0) = \alpha$$

In addition, we seek schemes for which the difference solution is guaranteed to be positive. We have found a first order scheme and a second order scheme for approximating (1) with these properties. In addition we

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have given a detailed error analysis for these schemes. The error analysis results in an error bound for the approximate solution of (1) that is uniformly valid for all positive D and h in a neighborhood of (0, 0). The error bound contains terms that measure the effect of the boundary layer and terms that reflect the accuracy of the reduced system of difference equations, considered as an approximation to the reduced problem (2).

Some preliminary numerical results indicate that the use of our second order scheme eliminates spurious oscillations in the approximate solution. A paper that represents the results is being written (B. Kellogg and A. Tsan).

(b) A polynomial approximation method of solving the kidney models was tested in a 4-tube model of the medulla that includes ascending and descending vasa recta, descending limb of Henle, and interstitium. In this method, interstitial concentrations and pressures are approximated by polynomials. The individual tubes are then solved against the interstitium with a relatively fine space chop. Solute and water accumulation in the interstitium is then computed. If this is more than some preset tolerance, the coefficient of the approximating polynomials are iteratively adjusted by a Newton-Raphson technique. When compared with the conventional trapezoidal scheme we have used in most of our calculations, the polynomial approximation method saved much storage space, some cpu time and decreased the oscillation in the transmural fluxes for comparable truncation error (Tsan).

(c) Other higher order schemes were tested. These included cubic and fifth degree overhang and a corrected trapezoidal rule. These gave a smaller truncation error than the conventional trapezoidal scheme for smooth data, but require special treatment in the vicinity of discontinuities such as the transition from the inner to the outer medulla. As a part of this work, a vastly improved error analysis of the trapezoidal method, based on the work by Keller (SIAM J. Numer. Anal. 11:305-320) on two point boundary value problems was carried out (with Tewarson).

Proposed Course:

The multinephron model has reached a level of sophistication such that quantitative simulation of certain aspects of renal function seems feasible. We have already begun an analysis of urea cycling and plan to complete this. The application of the model to various transient problems such as the transition of the kidney from diuresis to antidiuresis and the analysis of tracer washout curves is planned. We also plan to use the model to begin work on problems of system identification. In all of these applications, steady improvement of numerical methods is instantly utilized to give more accurate and faster computations with resulting increases in the size of the problems that can be analyzed for a given cost.

Publications:

Stephenson, J. L. Introductory remarks on the solution of kidney models. Conference Proceedings, 1976 Summer Computer Simulation Conference, Washington, D. C. pp. 451-455.

†Tewarson, R. P., Stephenson, J. L., Kydes, A. and Mejia, R. Use of sparse matrix techniques in numerical solution of differential equations for renal counterflow systems. Computers and Biomedical Research, 9:507-520, 1976.

Mejia, R., Stephenson, J. L., and Kellogg, R. B. Comparison of numerical methods in solving renal countercurrent equations. Conference Proceedings, 1976 Summer Computer Simulation Conference, Washington, D. C. pp. 502-506.

*Hubbard, B. E. Computing transient solutions for certain renal counterflow systems. Conference Proceedings, 1976 Summer Computer Simulation Conference, Washington, D. C. pp. 507-510.

Mejia, R., Kellogg, R. B., Stephenson, J. L., Comparison of numerical methods for renal network flows. J. Comp. Phys. 23:53-62, 1977.

Farahzad, P. and Tewarson, R. P., An efficient numerical method for solving the differential equations of renal counterflow systems. Computers in Bio. and Med. 1977 (forthcoming).

†Tewarson, R. P., Stephenson, J. L., Kydes, A., Mejia, R. Use of sparse matrix techniques in numerical solution of differential equations for renal counterflow systems. Computers and Biomedical Res. 9:507-520, 1976.

†Tewarson, R. P. Sparse matrix methods and mathematical models of the renal concentrating mechanism. Conference Proceedings, 1976, Summer Computer Simulation Conference, Washington, D. C. pp. 500-501.

†Tewarson, R. P., Stephenson, J. L., Juang, L. L. A note on solution of large sparse systems of nonlinear equations. J. Math. Anal. and Appl. 1977 (forthcoming).

†Supported in part by NHLBI under Contract 201HL03202.

*Supported by NHLBI under Contract HI 52900.

PROJECT NUMBER (Do NOT use this space)

HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

Z01 HL 03203-02 STB

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Theory of Epithelial Transport

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. Huss	NIH Postdoctoral Fellow
	A. Weinstein	Research Associate

COOPERATING UNITS (if any)

NONE

LAB/BRANCH

SECTION:

Section on Theoretical Biophysics

INSTITUTE AND LOCATION:

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	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)

The purpose of this project is to develop the theory of solute and water transport across epithelia. The project is directed particularly toward problems in proximal tubule transport in the mammalian kidney. Present studies center on the effect of changes in hydrostatic, osmotic, and protein oncotic pressure on the osmolality and flow rate of transported fluid in models that include both cellular and shunt pathways.

1058

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The purpose of this project is to develop the theory of solute and water transport across epithelia. The project is directed particularly at problems in proximal tubule transport in the mammalian kidney. Ultimately we hope to develop models that will account for steady state and time dependent transport of cations, anions, organic solute, and water through cellular and shunt pathways. Current studies center on the effect of changes in hydrostatic, osmotic, and protein oncotic pressure on the osmolality and flow rate of transported fluid.

Specific Findings: Work was continued on the model of flow in lateral intercellular channels. It was found that adding the cell as another element of the model, with flow and concentration gradients, yielded results substantially the same as those when the cell was considered to be isotonic with the luminal fluid. This is due, in large measure, to the assumption of large hydraulic and solute permeabilities of the apical cell membrane. However, with a single solute system, reducing these permeabilities results in an abnormally large negative hydrostatic pressure in the cell in order to maintain flow conservation (Huss).

Another version of the model that permits the simulation of transient behavior of the system is being developed. Again the epithelium is represented as a four compartment model - mucosal and serosal baths, cells and lateral interspaces, and the differential equations for volume flow, pressure, and non-electrolyte transport across the epithelium are solved by the same finite difference technique used for the kidney models (Weinstein).

A small side project on glomerular filtration was initiated. The results of recent experiments by Baylis, et al (Am. J. Physiol. 232(1):F58-F71, 1977) have been interpreted to indicate that the overall ultrafiltration coefficient (K_f) of the glomerular capillaries increases with the protein concentration of the arterial blood. Using a model developed earlier (Huss, et al, Ann. Biomed. Engr. 3:72-99, 1975), several hypotheses regarding the site of action of such changes were tested. Since the calculated value of K_f depended on the transmural hydrostatic pressure difference, and since the location in the capillary of the pressure probe was unknown, it was first postulated that the difference in K_f may be due to a steeper hydrostatic pressure gradient caused by the increased protein concentration. This was tested in the model. The results showed that if such an effect were to occur, the resulting change in GFR would be negligibly small, and furthermore would be in a direction opposite to that observed experimentally.

A second hypothesis was that the computed change in K_f may be due to a non-uniform hydraulic permeability along the capillaries. To test this, two distributions were used: one varying linearly from zero at the afferent end to a maximum at the efferent end, and one varying linearly from a maximum at

the afferent end to zero at the efferent end. The GFR for either of these cases was insignificantly different from the case of a uniform hydraulic permeability over the length.

A third hypothesis was that the number of capillaries in the bed open to flow is a function of the arterial protein concentration. Upon further examination of the reported experimental data, however, it was found that there was also a strong dependence of K_f on renal arterial blood flow. There is thus a possibility that the number of capillaries available increases as the arterial blood flow increases, and that the imputed dependence on protein concentration may be an artifact resulting from the correlation between blood flow and protein concentration in the experiments (Huss).

Proposed Course:

The model will be extended to include transport of electrolytes under an applied potential difference. Results of simulations with the model will be compared with actual data generated in another laboratory.

Annual Report of the
Section On Laboratory Animal Medicine and Surgery
Office of the Director of Intramural Research
National Heart, Lung, and Blood Institute
July 1, 1976 to September 30, 1977

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 rodent and acquatic 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 Building 3 in close proximity to the animal surgery laboratory. Postoperative intensive care and treatment of surgery patients is accomplished in this area. Large animals maintained following recovery from surgery for experimental protocols are held by VRB, DRS or at a contract site.

The animal surgery laboratory primarily supports the CHB, LEA, LTD and the Surgery Branch in preparation of experimental animal models and in collecting various biological specimens. The laboratory has a fully equipped x-ray catherization suite, clinical chemistry laboratory, three sterile operating suites, one acute operating suite and required support facilities in which more than 240 cardiopulmonary by-pass cases and 760 cases of thoracotomies, laparotomies, catheter implantations, thyroidectomies, caesarian sections, etc., were completed. Also, more than 400 procedures were performed in x-ray including 100 catherization-contrast media injection studies.

The NHLBI Sheep Colony NO1-HI-7-2900 has been in its fifth year of operation with continued success in breeding laboratory sheep 52 weeks per year at a rate to meet 12-month NHLBI and NICHHD laboratory requirements. More than 600 animals were delivered to meet laboratory requirements of gestation stages from 35-140 days, varied age and size lambs, and varied size young adult and aged sheep.

A contract with Flow Laboratories, Inc., NIH 263-76-C-0551 CC has been in effect four years primarily for boarding dogs on study requiring long-term holding. The development of a breeding colony of Newfoundland dogs has been phased in the last three years from breeding stock obtained from the School of Veterinary Medicine, University of Pennsylvania that originated from an inbred line of dogs effected with discrete subaortic stenosis. A colony of more than 70 dogs has been developed with approximately 50% demonstrating systolic murmurs of varying degrees of severity. Sufficient numbers of offspring are presently being produced to allow availability of affected animals to IR laboratories after January 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03401-01 LAMS

PERIOD COVERED
July 1, 1976 to September 30, 1977

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

COOPERATING UNITS (if any)

LAB/BRANCH
Office of the Director of Intramural Research

SECTION
Section on Laboratory Animal Medicine and Surgery

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20014

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)

The Newfoundland Breeding Colony maintained by Flow Laboratories, Inc., NIH 263-76-C-0551 CC has been developed from 15 dogs affected with varying degrees of discrete subaortic stenosis. More than 50% of dogs produced have developed varying degrees of severity of this defect with terminal cases resulting in sudden death.

Cardiac catherizations have demonstrated peak systolic pressure gradients between left ventricle and aorta from 35 to 150 mm Hg in affected animals.

Necropsy findings demonstrated severe left ventricular hypertrophy, discrete subaortic stenosis, severe abnormality of intramural coronary arteries in the ventricular septum, minor degrees of myocardial fiber disorientation and in several animals infective endocarditis.

Maintenance of animal breeding and rearing of puppies is complicated by the necessity of artificial insemination of females and the artificial rearing of newborn pups. Sufficient numbers of animals are being bred to allow availability of this animal model for IR research programs in the near future.

1061

Project Description:

The Newfoundland Breeding Colony has been developed as a source of dogs with hereditary subaortic stenosis and other defects that may spontaneously occur. An attempt has been underway to produce sufficient numbers of affected dogs that would adequately support acute and chronic research protocols of drug therapy, biochemistry, physiology, embryology, pathology and surgery. A major effort is required with varying degrees of success to produce animal models using experimental surgery in normal dogs that are similar to conditions that occur in human patients. This naturally occurring animal model would be more appropriate to use in such experiments.

Discrete subaortic stenosis has been studied extensively 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. Unfortunately, the trait appeared in early development of the Newfoundland breed and went undetected until substantially distributed.

Approximately 50% of colony offspring produced thus far demonstrate systolic murmurs of varying intensity. Cardiac catherization of more than 25 dogs showed peak systolic blood pressure gradients between left ventricle and aorta ranged from 35 to 150 mm Hg in those demonstrating Grade II-V murmurs.

Necropsy examinations were made by the Laboratory of Pathology, IR, on dogs that died suddenly and those studied acutely. All specimens had discrete subaortic narrowing located 8-10 mm below the level of the most caudal extension of the aortic valve cusps. The discrete narrowing was circumferential and extended transversely from the anterior mitral leaflet at the level of its attachment to the atrial septum, over the ventricular septum and the outflow surface of the leaflet. Concentric left ventricular hypertrophy occurred in all with the ventricular cavities of normal size with one exception. Abnormal intramural coronary arteries in the ventricular septum and minor degrees of myocardial fiber disorientation was present in all. Infective endocarditis occurred in 4 of 5 dogs that died spontaneously.

The colony has been maintained at Flow Laboratories, Inc., Dublin, Virginia. All animals are the property of NIH. The contractor is responsible for maintenance of all age groups, all breeding, whelping of 10-20% of litters and autopsy of animals lost at the contract site with delivery of preserved specimens to NIH.

Most of the pregnant bitches have been whelped in Building 3 or by Blanket Purchase Order Number PD 110722-7 (Donna Matthews) where survival rate of puppies has been 80% or higher.

All special studies including cardiac catherizations, pathology and classification of disease severity is the responsibility of the project officer.

The project officer has been responsible for monitoring the development of maintenance techniques used by the contractor for breeding adults and rearing newborn puppies. Problems have existed throughout but substantial improvement have been made the last half of the report period.

Artificial insemination is required because high incidence of hip dysplasia limits the ability of large dogs to breed naturally. Artificial rearing of all puppies has been necessary because of a high mortality rate of puppies raised with bitches from accidental death and agalactia.

These problems have been solved as indicated by the present 50-70% conception rate and 80-90% survival rate of hand reared puppies.

At the present rate of production, 100 dogs can be raised per year, 50-70 available for laboratory use and the remainder designated to colony replacements and losses.

The colony will be maintained in size and productivity as required by future IR laboratory demand.

Contract Information:

Contract Number NIH 263-76-C-0551 CC

Total Services - $135,000 - 14 months

Newfoundland Colony - $50,000

Contractor: Flow Laboratories, Inc.

Dublin, Virginia

PI: W. Knapp

A. Irwin

(est) Manyears: Professional - 0.2

Other - 3.8

Total - 4.0

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03402-01 LAMS

PERIOD COVERED
July 1, 1976 to September 30, 1977

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)

R. Chez PRB, NICHHD

LAB/BRANCH
Office of Director of Intramural Research

SECTION
Section on Laboratory Animal Medicine and Surgery

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20014

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)

The Laboratory Sheep Colony, Contract Number NO1-HI-7-2900 in its fifth year, has continued to meet specific animal model requirements of IR laboratories including principally CHB, LTD and SB, as well as NICHHD, IRP laboratories including PRB and LBS.

Practices that have contributed to the reduction of undesired production variables include: (1) continuous prophylactic immunization of various age groups; (2) accurate pregnancy diagnosis using Doppler ultrasound; (3) monitoring of animal health using various diagnostic laboratory techniques and (4) many other husbandry techniques unique to this colony.

Such practices are cost prohibitive in commercial sheep flocks which results in an unreliable supply of disease-free animals that meet the requirements of biomedical research.

1064

Project Description:

The breeding colony was originally developed as a source of sheep with AA type hemoglobin to meet requirements of MHB, IR. Since other laboratory programs were dependent on commercial suppliers for delivery of young lambs and pregnant ewes with a rate of 40-60% acceptable animals, the colony was gradually increased in size and productivity to meet additional requirements. From 850 to 950 varied age sheep exist in the colony year-round. More than 620 animals were delivered to NIH and other facilities for laboratory use during the report period.

The project officer has been responsible for development and updating of guidelines used by the contractor to meet NIH laboratory requirements. The contractor has been responsible for developing and updating husbandry techniques that allow natural breeding and management practices at his facility that meet laboratory requirements. Health care of colony sheep (property of NIH) has been assumed by the project officer throughout to provide optimal conditions of all age groups and to allow substantial preconditioning of all animals for delivery to laboratories from one-month of age.

Immunization protocols require vaccination by contract personnel of all lambs with ovine and bovine bacterins, and tetanus toxoid with biweekly boosters of each by the fifth week of age. This practice has essentially eliminated enterotoxemia and 98% of chronic pneumonia previously experienced. Booster injections of these and other biologicals are administered at later dates to various age and reproductive groups to facilitate adequate long-life protection of all animals.

The necessity of an accurate method of pregnancy diagnosis was determined at the onset of breeding five years ago because of natural seasonal variations of conception that range from 10 to 100%. Lindahl's technique using Doppler ultrasound with rectal examination has been used since it allows a 100% accurate positive diagnosis in detected pregnant sheep at 25-35 days gestation. Negative animals, however, are re-examined at least twice. Examinations are performed weekly by contract personnel with more than 1,500 exams completed per year to detect approximately 500 pregnant ewes conceived over a 52 week period. Future attempts will be made to develop a practical screening method for identifying the presence of single and multiple fetuses at 25-35 days gestation using Time-mode or B-mode ultrasound by the project officer. Such a technique would reduce by 50% the number of sheep delivered to NIH for x-ray exam.

Laboratory tests are continuously performed to monitor flock health. Contract personnel monitor 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 states.

Serological screening of suspected disease entities is attempted when suspicious signs exist. Stored as well as recently collected serum has been tested by the Animal Disease Center, Ames, Iowa, for Blue Tongue and Chronic Progressive Pneumonia viral titers.

Serum from several aborted ewes was recently screened for Toxoplasma gondii and 5 of 6 samples found positive. Since identification of the existance of this problem, a collaborative effort with LPD, NIAID has been underway to identify the infection that continues in the breeding groups.

Many other husbandry techniques are in use to overcome seasonal conditions that effect natural year-round production of sheep.

The colony represents the "sole" source of laboratory sheep available 12 months a year to meet the various requirements of research programs supplied. Husbandry techniques are practiced 365 days per year to meet these requirements.

This project will continue with expectation that future requirements of IR programs and other NIH programs will continue to be met. Production goals and total numbers of animals maintained will be varied as required by changing laboratory demand.

Contract Information:

Contract Number NO1-HI-7-2900 - $228,000 - 12 months

Contract Site: Double J Farms
Luray, Virginia

PI: Jesse Judy - Contractor

Donna Matthews - Colony Manager

Total Manyears - 6.5

Professional - 2.0

Other - 4.5

ANNUAL REPORT OF THE
LABORATORY OF BIOCHEMICAL GENETICS
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
July 1, 1976 through September 30, 1977

Little is known at the molecular level about the process of synapse formation and the basis for the specificity of synaptic connections between cells. Most investigators assume that receptors are present on the surface of neurons and their synaptic partners which must interact with specificity before synapses can form; i.e., molecules which determine the specificity of synaptic connections. During previous years we focused on the problem of obtaining homogeneous populations of neurons which form synapses. Since normal neurons do not divide and since the number of cell types in the nervous system is not known and adequate criteria for defining cell type have not been established, clonal lines of neuroblastoma cells and somatic cell hybrids derived from neuroblastoma cells were generated and characterized with respect to components known to be required for synaptic communication, such as neurotransmitters, receptors, and action potential ionophores. Fusion of mouse neuroblastoma cells with rat glioma cells yielded a clonal hybrid cell line, NG108-15, which synthesizes, stores, and excretes acetylcholine and forms synapses with mouse striated muscle cells, properties not detected with parental cell lines. The clonal hybrid cell line provided an opportunity to test the specificity of synapse formation for if the formation of synaptic connections between neurons and muscle cells were dependent upon interactions between cell recognition molecules, then discrete classes of muscle cells with different specificities for synapse formation might be expected. During the past year, we showed that NG108-15 hybrid cells form synapses in abundance with cells from different muscles and from the chick, mouse and rat. Thus, only one class of striated muscle cells was detected with respect to the formation of synapses. A hypothesis was proposed that part of the specificity of the normal neuromuscular synapse is acquired after the synapses form by a selection process which terminates some synapses.

A clonal line of mouse myoblasts, G8, was established which forms well-differentiated striated muscle cells in vitro and forms synaptic connections with NG108-15 cells. The process of synapse formation now can be studied with two clonal cell lines. G8 muscle cells were highly sensitive to acetylcholine at all sites tested on the surface of the myotubes, whereas, primary mouse myotubes were highly sensitive to acetylcholine only at sharply localized sites on myotube membranes estimated to <1% of the myotube surface membrane. G8 muscle cells thus are defective with respect to localization of nicotinic acetylcholine receptors.

Other cell lines of neural origin were obtained which form synapses with striated muscle cells. In addition, some cell lines were found which synthesize acetylcholine and adhere well to muscle cells but do not form synapses with muscle cells. The ability to form synapses and the synapse defects are stable properties which are inherited in a clonal fashion. Current studies focus on defining the reactions which are required for synapse formation and those which may be lacking in synapse defective cell lines.

Neurons dissociated from chick embryo retina were cultured. The cells synthesize nicotinic and muscarinic acetylcholine receptors, and possess high acetyltransferase and glutamic acid decarboxylase activities. The cultured cells form approximately 1.5 x 10^9 synapses per mg of protein which corresponds to approximately 60 synapses per average neuron. At least 3 types of synapses were detected which closely resemble those of the intact retina. Retina neurons also form synapses with striated muscle cells. The mismatched, inappropriate synapses between retina neurons and myotubes are terminated slowly over a period of 11 days. During this time, synapses between retina neurons become more abundant. These results show that retina neurons form transient synapses which turn over, and that the rate limiting step in synapse turnover is the termination, rather than the formation, of the synapse. In contrast, the termination of synapses between NG108-15 cells and striated muscle cells was not detected. Evidence was obtained for a reaction which is required for synapse formation after neurons adhere to muscle cells and for another reaction which is required for synapse termination.

Methods were devised for measuring fmol quantities of [^{3}H]-acetylcholine released by NG108-15 cells. Serotonin or $PGF_2\alpha$ which depolarize NG108-15 cells were found to stimulate acetylcholine release from the cells; whereas PGE_1 had little or no effect. Activation of the Na^+ action potential ionophore with veratridine or the addition of KCl also evoked acetylcholine release from cells. The ability of cells to store and release acetylcholine was found to be regulated. Methods were found for shifting populations of cells from a competent state with respect to the storage and release of acetylcholine to a defective state and vice versa. In consequence, synapse formation by clonal cells can be regulated.

Chick embryo retina was found to be a rich source of both muscarinic and nicotinic acetylcholine receptors. Both muscarinic and nicotinic acetylcholine receptors are synthesized before synapses appear in the retina; however, during development, nicotinic acetylcholine receptors become associated predominantly with neurites in the synaptic layers of the retina. Muscarinic acetylcholine receptors also were found to localize in the inner synaptic layer of the retina, but the receptor distribution differs from that of nicotinic acetylcholine receptors. The properties of muscarinic acetylcholine receptors were determined at different developmental ages and were compared with the properties of muscarinic inhibitory and excitatory receptors of neuroblastoma and hybrid cells.

Two pathways for γ-aminobutyric acid synthesis were found in chick embryo retina. The first pathway depends upon the conversion of putrescine to ornithine and the subsequent conversion of ornithine to γ-aminobutyric acid. The second route of synthesis is dependent upon the conversion of glutamic acid to γ-aminobutyric acid, catalyzed by glutamic acid decarboxylase. Elevation of cAMP levels in neuroblastoma cells was shown to induce ornithine decarboxylase activity. Thus, in the developing embryo, neurotransmitters which affect cAMP levels may regulate ornithine decarboxylase activity and thereby control the rate of GABA synthesis from ornithine.

GABA was found to regulate the specific activity of glutamic acid decarboxylase in cells dissociated from chick embryo retina and cultured in vitro.

Endorphin peptides were shown to inhibit adenylate cyclase of NG108-15 cells. The inhibition constants (K_i) were 12, 40, 63 and 98 nM for methionine-enkephalin, leucine-enkephalin, β-endorphin and α-endorphin, respectively. Thus, the endorphins are the most potent peptide inhibitors known and thus, the activations of adenylate cyclase by other species of neurotransmitters for hormones are suppressed. In effect, the opiate peptides act as pleitropic desensitizers of many kinds of receptors, which in concert with the corresponding ligands, activate adenylate cyclase. Exposure of cells to methionine-enkephalin for 12 to 97 hours results in an increase in adenylate cyclase activity which compensates for the inhibition of enzyme activity by methionine-enkephalin. The cells then have normal cAMP levels and appear tolerant to methionine-enkephalin because the increase in adenylate cyclase activity is approximately equal to the inhibition of enzyme activity by the peptide. However, the cells are dependent upon the opiate to maintain normal cAMP levels. Withdrawal of methionine-enkephalin removes the enzyme inhibition and reveals abnormally high adenylate cyclase activity. Dual regulation of adenylate cyclase by opiates thus accounts for the phenomena of narcotic dependence and tolerance. Thus, the endorphin peptides and narcotics are pleitropic regulators of cell responses to neurotransmitters and hormones which are coupled to the activation of adenylate cyclase. In this way, opiates can alter the perception of neurons to incoming messages which are destined for adenylate cyclase.

Reactions mediated by the opiate receptors that inhibit adenylate cyclase are closely coupled to subsequent reactions that gradually increase adenylate cyclase activity of neuroblastoma x glioma NG108-15 hybrid cells. Opiate-treated cells have higher basal-, PGE_1-, and 2-chloroadenosine-stimulated activities than control cells. However, NaF or guanosine 5'(β,γ-imido)triphosphate abolish most of the difference in adenylate cyclase activity observed with homogenates from control and opiate-treated cells. Cycloheximide blocked some, but not all, of the opiate-dependent increase in adenylate cyclase activity. These results suggest that the opiate-dependent increase in adenylate cyclase is due to conversion of adenylate cyclase to a form with altered activity. Protein synthesis also is required for part of the opiate effect. A hypothesis is proposed that the activity of adenylate cyclase determines the rate of conversion of the enzyme from a high to a low activity form or by inhibiting adenylate cyclase.

Highly purified [Leu^5]enkephalin and seven derivatives including [Ala^2,Leu^5]-, [Ser^2,Leu^5]-, [Ser^3,Leu^5]-, [Aba^2,Leu^5]-, and [des-$Gly^{2(3)}$,Leu^5]enkephalin were obtained by solid phase synthesis and their morphine-like activities in neuroblastoma x glioma cell homogenates were all less active than [Leu^5]enkephalin. The results are discussed in terms of recently suggested conformational structures for the enkephalin peptides. No melanocyte stimulating activity was observed for [Leu^5]enkephalin, [Ala^2,Leu^5]enkephalin, or [Ser^2Leu^5]enkephalin.

NG108-15 hybrid cells also possess depolarizing alpha receptors and muscarinic acetylcholine receptors which also are coupled to the inhibition of adenylate cyclase. Cells were cultured in the presence of norepinephrine or carbamylcholine for 10 to 48 hours, then the effects of withdrawal of the receptor ligand either by replacing the medium or by the addition of a receptor

antagonist was tested. Withdrawal of norepinephrine or carbamylcholine resulted in 9- and 5-fold increases in cAMP levels of intact cells, respectively. Adenylate cyclase activity also increased but to a lesser extent. Studies on the specificity of receptor activators and antagonists showed that the inhibitons of adenylate cyclase by norepinephrine, acetylcholine and opiates are mediated by different species of receptors. These results show that dual regulation of adenylate cyclase is a general phenomenon mediated by different species of receptors and that cells can become dependent upon norepinephrine or acetylcholine, as well as opiates. Cells develop an apparent tolerance to these compounds but in fact are responsive to the compound used.

A clonal cell line (AtT20) derived from a mouse pituitary tumor was found which synthesizes endorphin peptides with opiate activity. Fractionation revealed 4 species of endorphins; the most abundant species with apparent molecular weights of 1800 and 2400 were purified 300- and 24-fold, respectively. Additional minor components were found with apparent molecular weights of >3000 and >750. The endorphins differed in sensitivity to inactivation by proteolytic enzymes. Endorphin activity was destroyed by treatment with cyanogen bromide which suggests that the endorphins are derived from an inactive prohormone, β-lipotropin, by cleavage at specific sites. The concentrations of endorphins and endorphin precursor proteins in AtT-20 cells was estimated to be 5% of the total cell protein. Thus, AtT-20 cells can be used to study endorphin synthesis and conversion from inactive to active forms and regulation of endorphin secretion from the cell.

[^{3}H]-Quinuclidinyl-benzilate (QNB) and [^{3}H]-scopolamine were used to study excitatory acetylcholine receptors of neuroblastoma x glioma NG108-15 cells and inhibitory acetylcholine receptors of clonal neuroblastoma N1E-115 cells. Both ligands bind with high affinity to muscarinic acetylcholine receptors of the cells. The apparent dissociation constants are 0.1 nM (NG108-15) and 0.06 nM (N1E-115) for [^{3}H]QNB; and 0.5 nM (NG108-15) and 0.4 nM (N1E-115) for [^{3}H]-scopolamine.

If each muscarinic acetylcholine receptor molecule contains one specific binding site for QNB, then the average NG108-15 or N1E-115 cell possesses 30,000 and 22,500 acetylcholine receptors/cell, respectively. Activation of one inhibitory acetylcholine receptor of N1E-115 cells results in the synthesis of approximately 20,000 molecules of cGMP in 15-20 sec (670 cGMP molecules synthesized/sec/receptor), cell hyperpolarization (usually -15 mV for 10-15 sec), and desensitization of the receptors; whereas, activation of one excitatory muscarinic acetylcholine receptor of NG108-15 cells results in the synthesis of <1 molecule of cGMP/receptor/sec, depolarizes the cell and desensitizes the receptor within 30 sec with respect to cGMP accumulation and ionophore activity. The results suggest that the desensitized form of the receptor concomitantly converts the receptor to a form which is a potent inhibitor of adenylate cyclase. The receptor mediated inhibition of adenylate cyclase is dependent on a ligand which activates receptors, is blocked by receptor antagonists, but is not desensitized by receptor activators. Hill coefficients of approximately 1 were found for receptor antagonists and approximately 0.5 for receptor activators. Antagonist binding therefore can be described as interaction of ligand with an independent, noninteracting class of receptors whereas

binding of receptor activators exhibits either negative cooperativity or heterogeneity of binding sites. [Ligand·Receptor] heterogeneity was demonstrated by analysis of the kinetics of [^{3}H]-QNB association with and dissociation from the receptors revealed 3 sequential reactions which affect the properties of the [carbamylcholine·receptor] complexes formed. The functional coupling of the [activator·receptor] complex with the adenylate cyclase complex provides further evidence for multiple forms of [ligand·receptor].

The receptors from both cell lines were solubilized from membranes and 2 methods of assaying the solubilized receptors were devised. The apparent negative cooperativity of the [activator·receptor] complex was abolished by solubilization. A sequence of reactions was proposed concerning the mechanism of receptor activation, desensitization and the actions of receptors based on the different forms of [ligand·receptor] complex and the reactions which were found.

Further studies were made of the muscarinic acetylcholine receptors in unresponsive embryonic chick hearts (day 3 in ovo) and responsive chick hearts (day 9 in ovo). Extensive analysis of ligand binding by active and inactive receptors revealed that a small time-dependent increase in the affinity of carbamylcholine for the receptor was observed as the only difference. Thus, the physiologically inactive receptors in young embryonic hearts must be defective in some aspect of receptor function subsequent to ligand binding.

Kinetic analysis of QNB binding in heart was undertaken in view of the previous observation (project report no. Z01 HL 00012-02 LBG) that binding occurs in a two step reaction to receptors of brain and neuroblastoma and hybrid cells. We confirm that, in heart, QNB first forms a rapidly reversible complex with the muscarinic receptor followed by a more slowly reversible complex. Kinetic data conclusively show that this is a two step sequential reaction. It is likely that it represents a QNB induced conformational change in the receptor. This conformational change occurs in both active and inactive receptors.

The binding of ^{125}I-labelled derivatives of scorpion toxin to the action potential Na^+ ionophores was studied thoroughly using two derivatives, a [^{125}I]iodotyrosyl derivative prepared by lactoperoxidase catalyzed iodination and a 3-(3[^{125}I]iodo 4 hydroxyphenyl)propionyl lysyl derivative. Pure monosubstituted and disubstituted derivatives were obtained by column chromatography. Both these derivatives retain full biologic activity. Both these derivatives bind to a single class of saturable sites in electrically excitable neuroblastoma cells. The density of these sites is 22000/cell or 25/μm^2 of surface membrane and their dissociation constant is 1 to 2 nM. No saturable binding sites were observed in variant neuroblastoma cells which specifically lack the action potential Na^+ response. These and other results demonstrate that the toxin binds specifically to the Na^+ ionophore.

Binding of scorpion toxin is inhibited by depolarization of the cells. The inhibition is due to an increase in K_D (10 fold per 32 mV) with no change in the number of bindings sites. These results suggest that depolarization causes a conformational change in the scorpion toxin receptor site that results in a reduced affinity for scorpion toxin. Binding is also inhibited by polypeptide toxins isolated from sea anemone indicating that these toxins, which

have a similar physiologic effect, act at the same receptor site as scorpion toxin.

Our previous results showed that the receptor site for the alkaloid toxins veratridine, batrachotoxin, aconitine and grayanotoxin and the receptor site for scorpion toxin interact cooperatively in activating the action potential Na^+ ionophore. We have now developed an allosteric model which quantitatively describes this interaction. This model assumes that alkaloid toxins activate the action potential Na^+ ionophore by binding better to the active state of the ionophore and that scorpion toxin reduces the energy required for activation of the ionophore by alkaloid toxins.

Saxitoxin and tetrodotoxin are noncompetitive inhibitors of activation by alkaloid toxins and scorpion toxin. Purified ^3H-labelled saxitoxin binds to a single class of sites in neuroblastoma cells. These sites are not membrane potential dependent. The K_D of these sites is 3 nM and there are approximately 60,000 sites per cell or about 3 saxitoxin sites for each scorpion toxin site. Thus, either there are Na^+ ionophores which bind saxitoxin but not scorpion toxin or each Na^+ ionophore has three saxitoxin receptor sites and one scorpion toxin receptor site. These findings have important implications for the allosteric properties of the Na^+ ionophore.

Our studies have now defined three specific neurotoxin receptor sites associated with the action potential Na^+ ionophore. Each of the 8 neurotoxins that effect the ionophore interacts specifically with one of these sites. These sites are likely to be located on important functional regions of the ionophore.

Yohimbine, a drug with both local anesthetic and antiarrhythmic properties, inhibits activation of the action potential Na^+ ionophore by alkaloid toxins. The inhibition is competitive with respect to both batrachotoxin and veratridine. These observations suggest that the receptor site for alkaloid toxins may also be a locus of local anesthetic and antiarrhythmic action. These drugs may therefore be allosteric effectors of the Na^+ ionophore.

The selectivity of transport by the acetylcholine receptor Na^+ ionophore was studied. Two series of labelled compounds of similar size but differing in charge and chemical groupings were used: (1) quanidine (+1), formamide (uncharged), and urea (uncharged); (2) ethylamine (+1), ethylene diamine (+2,+1,0 depending on pH), ethanolamine (+1,0 depending on pH), and ethylene glycol (uncharged). All of these compounds were detectably permeant. Two generalizations can be made. Monovalent compounds were more permeant than divalent or uncharged compounds of the same size. Substitution of hydrogen bond donating groups like -OH increased permeability. The results suggest a strong effect of chemical interactions of permeant molecules with the ion transport channel in determining selectivity.

The alkaloid neurotoxin histrionicotoxin inhibits nicotinic acetylcholine receptors. Unlike other inhibitors such as d-tubocurarine and α-bungarotoxin, its inhibition is not competitive with acetylcholine. In fact, histrionicotoxin increases the affinity of the receptor for acetylcholine and carbamylcholine.

Desensitization of the receptor also increases its affinity for acetylcholine. Therefore, our results suggest that histrionicotoxin inhibits receptor function by causing premature desensitization by binding preferentially to the desensitized state of the receptor and stabilizing it.

In E. coli, the phenomenon of catabolite repression by sugars is correlated with the capacity of such sugars to inhibit adenylate cyclase. The mechanism by which sugars inhibit adenylate cyclase is complex, since glucose inhibits the enzyme in intact cells, but not in cell-free extracts. It has been established that a functional sugar transport system is necessary for glucose to exercise inhibition of adenylate cyclase. The phosphoenolpyruvate-dependent sugar transport system, known at the PTS, appears to interact with adenylate cyclase to form a functional multi-enzyme complex that responds to regulation by sugar substrates of the PTS. Mutants in the membrane-bound specific permeases for α-methylglucoside or 2-deoxyglucose render adenylate cyclase insensitive to inhibition by those sugars and eliminate transport activity for those sugars. Other studies with wild-type cells using analogs of glucose established that the stereochemical specificity for sugar inhibition of adenylate cyclase matches that for sugar transport by the specific permeases. These studies demonstrate that sugar inhibition of adenylate cyclase requires an interaction of the sugar with the cell-surface permease. Other studies indicate that mutants in the PTS protein known as Enzyme I have low adenylate cyclase activity and that phosphoenolpyruvate-dependent phosphorylation of PTS proteins is responsible for the activation of adenylate cyclase. Taken together, these results provide support for the model that sugars inhibit adenylate cyclase by a mechanism involving a displacement of the equilibrium of PTS proteins from a phosphorylated to a dephosphorylated state.

Chicken ovalbumin is a protein characterized by a blocked amino-terminus: N-acetylglycine. A long-standing area for investigation has concerned the mechanism by which such unusual terminal amino acids are formed. Previous studies on immunoglobulins which contain N-terminal pyroglutamate have indicated that the proteins are synthesized as precursors containing N-terminal methionine. Processing of the precursor by some unknown mechanism results in a protein containing N-terminal pyroglutamic acid. The present experiments used the same approach in a study of ovalbumin synthesis. Pulse-labeling studies with radioactive methionine established that ovalbumin is also synthesized as a precursor containing N-terminal methionine. Therefore, it appears likely that all proteins containing N-blocked amino-termini may be synthesized as larger precursors that are subsequently degraded and modified to form amino-termini such as pyroglutamic acid or N-acetylglycine.

Thyrotropin releasing hormone (TRH) is a modified tripeptide which is found in brain and which has a variety of unique biological functions. It has previously been shown that an enzyme with amidase activity converts TRH to pyroglutamyl-histidyl-proline (acid TRH) that thus far has been shown to have none of the biological properties of TRH. It has therefore been assumed that the pathway for metabolism of TRH involves this single mode of degradation and that the metabolic product is biologically inactive. Our studies have uncovered the presence in hypothalamic extracts of two enzymes that metabolic TRH. The previously described amidase was found as a soluble enzyme which

showed the interesting property of being inhibited by thyroid stimulating hormones. Additionally, another soluble enzyme (a pyroglutamyl peptidase) that converts TRH to histidyl-prolineamide was identified. Of interest is the observation that the pyroglutamyl peptidase activity is inhibited by hydrocortisone. The discovery of a new metabolite of TRH (histidyl-prolineamide) suggests the possibility that this compound may be biologically significant. The examination of this possibility will be the thrust of future studies in this area.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 00001-05 LBG

PERIOD COVERED
July 1, 1976 through September 30, 1977

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

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3.5	2.5	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Our aim is to study the distribution of nicotinic acetylcholine receptors in intact and cultured tissues of the peripheral and central nervous system in relationship to the development and function of synapses. To this purpose histochemical localization of α-bungarotoxin bound to the receptors is used in conjunction with light and electron microscopy. In the past year we have used an α-bungarotoxin-horseradish peroxidase conjugate to identify the synaptic sites of nicotinic acetylcholine receptors in the chicken retina and studied the control of nicotinic acetylcholine receptor aggregation on cultured skeletal muscle cells by neuroblastoma-glioma hybrid cells and by substances secreted by these cells.

PHS-6040
(Rev. 10-76)

Project Description:

Methods Employed: We have used indirect immunoperoxidase staining of monolayer cultured cells to which αBT has been bound, and peroxidase staining of tissue incubated in vivo with peroxidase-labeled αBT. These materials are subsequently examined by light and electron microscopy.

Major Findings: (1) Horseradish peroxidase was crosslinked to αBT to form a conjugate which retained the specific affinity of αBT for nicotinic acetylcholine receptors. This conjugate bound to 5-7% of the synapses in the inner synaptic layer of the chicken retina. Amacrine cell and bipolar cell synapses bound conjugate, indicating that some synapses of both types have nicotinic acetylcholine receptors. (2) Co-culture of mouse muscle fibers with neuroblastoma-glioma hybrid cells (which form synapses with the muscle cells) increased the number of nicotinic acetylcholine receptor clusters 2-4 fold. A similar increase was obtained by adding cell-free conditioned medium from hybrid cell cultures to the muscle cell cultures. The effect did not depend on the synthesis of new receptors.

Significance to Biomedical Research: Knowledge of ultrastructural distribution of acetylcholine receptors is of clear importance in any attempt to understand the role of neurotransmitters and their receptors in the function and development of the nervous system. The α-bungarotoxin-immunoperoxidase technique already has shown promise for the diagnosis and analysis of mechanisms in human neuromuscular disorders.

The results obtained with chick retina (1) represent the first direct demonstration of the synaptic localization of neurotransmitter receptors in the central nervous system, and should lead to a better understanding of neuronal specificity in the CNS.

The cultured muscle studies (2) may lead to a better understanding of the mechanism whereby neurons control the distribution of receptors on muscle cells and on other neurons.

Proposed Course: (1) We will extend the study of cholinergic synapses in retina to: a) further analyze the pattern of cholinergic synaptic transmission, b) follow the course of receptor accumulation at synapses during development. (2) We will attempt to characterize the factor(s) in hybrid cell conditioned medium which promotes the aggregation of receptors on muscle cell membranes and to determine its mechanism of action.

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1. Ringel, S. P., Bender, A. N., Engel, W. K., Daniels, M. P. and Vogel, Z.: A sequential study of denervation-ultrastructural immunoperoxidase localization of alpha-bungarotoxin. Trans. Am. Neurol. Assoc. 100: 52-56, 1975.

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3. Carpenter, David O., Greene, Lloyd A., Shain, William and Vogel, Zvi: Effects of eserine and neostigimine on the interaction of α-bungarotoxin with Aplysia acetylcholine receptors. Mol. Pharmacol. 12: 999-1006, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00002-04 LBG

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Morphine Receptors as Regulators of Adenylate Cyclase

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)

The objectives are to elucidate the mechanisms of dependence upon opiates and to define the effects of opiate peptides on cells.

1078

Project Description:

Endorphin peptides were shown to inhibit adenylate cyclase of NG108-15 cells. The inhibition constants (K_i) were 12, 40, 63 and 98 nM for methionine enkephalin, leucine enkephalin, β-endorphin and α-endorphin, respectively. Thus, the endorphins are the most potent peptide inhibitors known and thus, the activations of adenylate cyclase by other species of neurotransmitters or hormones are suppressed. In effect, the opiate peptides act as pleitropic desensitizers of many kinds of receptors, which in concert with the corresponding ligands, activate adenylate cyclase. Exposure of cells to methionine enkephalin for 12 to 97 hours results in an increase in adenylate cyclase activity which compensates for the inhibition of enzyme activity by methionine enkephalin. The cells then have normal cAMP levels and appear tolerant to methionine enkephalin because the increase in adenylate cyclase activity is approximately equal to the inhibition of enzyme activity by the peptide. However, the cells are dependent upon the opiate to maintain normal cyclic AMP levels. Withdrawal of methionine enkephalin removes the enzyme inhibition and reveals abnormally high adenylate cyclase activity. Dual regulation of adenylate cyclase by opiates thus accounts for the phenomena of narcotic dependence and tolerance. Thus, the endorphin peptides and narcotics are pleitropic regulators of cell responses to neurotransmitters and hormones which are coupled to the activation of adenylate cyclase. In this way, opiates can alter the perception of neurons to incoming messages which are destined for adenylate cyclase.

Reactions mediated by the opiate receptors that inhibit adenylate cyclase are closely coupled to subsequent reactions that gradually increase adenylate cyclase activity of neuroblastoma x glioma NG108-15 hybrid cells. Opiate-treated cells have higher basal-, PGE_1-, and 2-chloroadenosine-stimulated activities than control cells. However, NaF or guanosine 5'-(β,α-imido)triphosphate abolish most of the difference in adenylate cyclase activity observed with homogenates from control and opiate-treated cells. Cycloheximide blocked some, but not all, of the opiate-dependent increase in adenylate cyclase activity. These results suggest that the opiate-dependent increase in adenylate cyclase is due to conversion of adenylate cyclase to a form with altered activity. Protein synthesis also is required for part of the opiate effect. A hypothesis is proposed that the activity of adenylate cyclase determines the rate of conversion of the enzyme from a high to a low activity form or via a new opiate, by inhibiting adenylate cyclase.

Highly purified [Leu^5]enkephalin and seven derivatives including [Ala^2,Leu^5]-, [Ser^2,Leu^5]-, [Ser^3,Leu^5]-, [Aba^2,Leu^5]-, and [des-$Gly^{2(3)}$,Leu^5]enkephalin were obtained by solid phase synthesis and their morphine-like activities in neuroblastoma x glioma cell homogenates were measured. Changes at the 2, 3, and 5 positions of the enkephalin provided analogues which were all less active than [Leu^5]enkephalin. The results are discussed in terms of recently suggested conformational structures for the enkephalin peptides. No melanocyte stimulating activity was observed for [Leu^5]enkephalin, [Ala^2,Leu^5]enkephalin, or [Ser^2,Leu^5]enkephalin.

Significance to Biomedical Research. Effects of endogenous opiate peptides on adenylate cyclase activity was defined and molecular mechanisms for the phenomena of narcotic dependence and tolerance were proposed.

Proposed Course: Further studies on the regulation of adenylate cyclase by narcotics and endorphin peptides and the mechanism of coupling inhibition of adenylate cyclase with a subsequent increase in adenylate cyclase activity are in progress.

Publications:

1. Lampert, Arthur, Nirenberg, Marshall and Klee, Werner A.: Tolerance and dependence evoked by an endogenous opiate peptide. Proc. Natl. Acad. Sci. USA 73: 3165-3167.

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7. Sharma, Shail K., Klee, Werner A. and Nirenberg, Marshall: Opiate dependent modulation of adenylate cyclase. Proc. Natl. Acad. Sci. USA, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00004-04 LBG

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Studies of Action Potential and Receptor Ionophores

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)

The objective of this project is to understand the molecular basis of electrical excitability. Our current efforts are directed toward defining structure-function relationships of the action potential Na^+ ionophore using neurotoxins that interact with three specific receptor sites associated with the action potential Na^+ ionophore. We are also studying the nicotinic acetylcholine receptor with respect to its transport selectivity and its interaction with histrionicotoxin, a toxin that causes premature desensitization of the receptor. These studies will lead to new understanding of the molecular events involved in the function of these membrane macromolecules which are involved in information processing in the nervous system and beating in heart.

1081

Project Description:

Objectives: The objectives of this project are (1) to develop biochemical methods for study of action potential and receptor ionophores, (2) to use these methods to study the mechanism of action of these macromolecules at the cellular and membrane levels, and (3) to solubilize, purify, and characterize these ionophores at the molecular level.

Methods Employed: Biochemical assays which measure changes in passive Na^+ influx were used to study the acetylcholine receptor ionophore and the action potential Na^+ ionophore. Specific binding methods using ^{125}I labelled scorpion toxin, ^{125}I labelled α-bungarotoxin, and ^{3}H labelled saxitoxin to measure the density of receptors and ionophores and to study their properties.

Major Findings:

Action potential Na^+ ionophore. The binding of ^{125}I-labelled derivatives of scorpion toxin to the action potential Na^+ ionophores was studied thoroughly using two derivatives, a [^{125}I]iodotyrosyl derivative prepared by lactoperoxidase catalyzed iodination and a 3-(3[^{125}I]iodo 4 hydroxyphenyl)propionyl lysyl derivative. Pure monosubstituted and disubstituted derivatives were obtained by column chromatography. Both these derivatives retain full biologic activity.

Both these derivatives bind to a single class of saturable sites in electrically excitable neuroblastoma cells. The density of these sites is 22000/cell or 25/μm^2 of surface membrane and their dissociation constant is 1 to 2 nM. No saturable binding sites were observed in variant neuroblastoma cells which specifically lack the action potential Na^+ response. These and other results demonstrate that the toxin binds specifically to the Na^+ ionophore.

Binding of scorpion toxin is inhibited by depolarization of the cells. The inhibition is due to an increase in K_D (10 fold per 32 mV) with no change in the number of bindings sites. These results suggest that depolarization causes a conformational change in the scorpion toxin receptor site that results in a reduced affinity for scorpion toxin. Binding is also inhibited by polypeptide toxins isolated from sea anemone indicating that these toxins, which have a similar physiologic effect, act at the same receptor site as scorpion toxin.

Our previous results showed that the receptor site for the alkaloid toxins veratridine, batrachotoxin, aconitine and grayanotoxin and the receptor site for scorpion toxin interact cooperatively in activating the action potential Na^+ ionophore. We have now developed an allosteric model which quantitatively describes this interaction. This model assumes that alkaloid toxins activate the action potential Na^+ ionophore by binding better to the active state of the ionophore and that scorpion toxin reduces the energy required for activation of the ionophore by alkaloid toxins.

Saxitoxin and tetrodotoxin are noncompetitive inhibitors of activation by alkaloid toxins and scorpion toxin. Purified ^{3}H-labelled saxitoxin binds

to a single class of sites in neuroblastoma cells. These sites are not membrane potential dependent. The K_D of these sites is 3 nM and there are approximately 60000 sites per cell or about 3 saxitoxin sites for each scorpion toxin site. Thus, either there are Na^+ ionophores which bind saxitoxin but not scorpion toxin or each Na^+ ionophore has three saxitoxin receptor sites and one scorpion toxin receptor site. These findings have important implications for the allosteric properties of the Na^+ ionophore.

Our studies have now defined three specific neurotoxin receptor sites associated with the action potential Na^+ ionophore. Each of the 8 neurotoxins that effect the ionophore interacts specifically with one of these sites. These sites are likely to be located on important functional regions of the ionophore.

Yohimbine, a drug with both local anesthetic and antiarrhythmic properties, inhibits activation of the action potential Na^+ ionophore by alkaloid toxins. The inhibition is competitive with respect to both batrachotoxin and veratridine. These observations suggest that the receptor site for alkaloid toxins may also be a locus of local anesthetic and antiarrhythmic action. These drugs may therefore be allosteric effectors of the Na^+ ionophore.

Nicotinic acetylcholine receptors. The selectivity of transport by the acetylcholine receptor Na^+ ionophore was studied. Two series of labelled compounds of similar size but differing in charge and chemical groupings were used: (1) guanidine (+1), formamide (uncharged), and urea (uncharged); (2) ethylamine (+1), ethylene diamine (+2,+1,0 depending on pH), ethanolamine (+1,0 depending on pH), and ethylene glycol (uncharged). All of these compounds were detectably permeant. Two generalizations can be made. Monovalent compounds were more permeant than divalent or uncharged compounds of the same size. Substitution of hydrogen bond donating groups like -OH increased permeability. The results suggest a strong effect of chemical interactions of permeant molecules with the ion transport channel in determining selectivity.

The alkaloid neurotoxin histrionicotoxin inhibits nicotinic acetylcholine receptors. Unlike other inhibitors such as d-tubocurarine and α-bungarotoxin, its inhibition is not competitive with acetylcholine. In fact, histrionicotoxin increases the affinity of the receptor for acetylcholine and carbamylcholine. Desensitization of the receptor also increases its affinity for acetylcholine. Therefore, our results suggest that histrionicotoxin inhibits receptor function by causing premature desensitization by binding preferentially to the desensitized state of the receptor and stabilizing it.

Significance to biomedical research: The results provide new insights into the mechanism of action and regulation of membrane macromolecules involved in information transfer and processing in the nervous system and in maintenance of normal beating in heart.

Proposed course: This project will be terminated because the principal investigator has left NIH.

Publications:

1. Catterall, W. A.: Activation of the action potential Na^+ ionophore by neurotoxins: An allosteric model. J. Biol. Chem., in press, 1977.

2. Catterall, W. A.: Membrane potential dependent binding of scorpion toxin to the action potential Na^+ ionophore. Studies with a toxin derivative prepared by lactoperoxidase catalyzed iodination. J. Biol. Chem., in press, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00008-01 LBG

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Synthesis of opiate peptides by clonal cell lines

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

PI:	Steven L. Sabol	Research Associate	LBG NHLBI
OTHER:	Marshall Nirenberg	Chief, LBG	LBG NHLBI
	John McDermot	Visiting Fellow	LBG NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Biochemical Genetics

Section on Molecular Biology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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)

Clonal cell lines of neural or pituitary origin were assayed for the presence of peptides with opiate-like properties by determining the ability of peptides extracted from cells to inhibit adenylate cyclase of NG108-15 neuroblastoma x glioma hybrid cells. Opioid peptides were found in cells of the AtT-20 mouse pituitary tumor clonal cell line, which is known to synthesize ACTH. Preliminary characterization of these peptides suggests that they resemble α-, β-, and γ-endorphin and Met-enkephalin, which are opioid peptides recently found in brain and the pituitary and which are thought to be synthesized by specific cleavage of the peptide β-lipotropin. Ongoing studies include further characterization of the opioid peptides and investigations of the regulation of their biosynthesis and secretion.

1085

Project Description:

Objectives: Peptides with opiate activity properties termed endorphins which are synthesized in the brain and pituitary are endogenous ligands of the opiate receptor. Clonal cell lines of neural or pituitary origin were examined for the presence of opioid peptides in order to obtain homogenous cell populations which synthesize and secrete endorphins.

Methods Employed: Endorphin activity of cell extracts was assayed in a novel way, which involved quantitation of opiate-receptor specific (naloxone-reversed) inhibition of adenylate cyclase activity of NG108-15 neuroblastoma x glioma hybrid cell homogenates.

Major Findings: Of 34 cell lines tested, opiate activity was found only in the AtT-20 mouse pituitary tumor ACTH-secreting cell line and its variant D16. The content of endorphin of AtT-20 cells was found to be at least 100 pmol Met-enkephalin equivalents/mg protein. Gel filtration analysis revealed the presence of at least four species of endorphins. The two most abundant species, with apparent molecular weights of 1800 and 2400, were purified 300- and 24-fold, respectively. Additional minor components were found with apparent molecular weights of >3000 and >750. The endorphins differed in sensitivity to inactivation by proteolytic enzymes. The peptides were inactivated by cyanogen bromide treatment which suggests that a methionine residue is required for opiate activity. Subcellular fractionation studies suggest that endorphins may be packaged into secretory vesicles. Treatment of AtT-20 cells with dexamethasone reduced the intracellular endorphin content as well as the ACTH content, which suggests that synthesis of these peptides is regulated coordinately.

Significance to Biomedical Research: This is the first cell line to be found to synthesize endorphins. The concentrations of endorphins and endorphin precursor proteins in AtT-20 cells was approximately 5% of the total cell protein; thus AtT-20 cells can be used to define the mechanism of endorphin biosynthesis and regulation of endorphin secretion. The resemblance of AtT-20 cells to normal corticotrophs of the anterior pituitary gland suggests that the cell line is a useful model system to study possible synthesis and secretion of endorphins by corticotrophs. From the results one may predict that excess endorphin secretion may occur in conditions of excess ACTH secretion. Furthermore, a basic understanding of endorphin physiology may help elucidate the mechanism of narcotic addiction in man.

Proposed Course: 1. Further purification and characterization of AtT-20 endorphins. 2. Biosynthetic studies to define possible precursor-product relationships in the generation of endorphins. 3. Study of factors regulating synthesis and secretion of endorphins. 4. Examination of other ACTH-secreting clonal cell lines and tumors, including human tumors, for possible production of endorphin peptides.

Publications:

1. Giagnoni, Gabriella, Sabol, Steven L. and Nirenberg, Marshall: Synthesis of opiate peptides by a clonal pituitary tumor cell line. Proc. Natl. Acad. Sci. USA 74: 2259-2263, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00009-03 LBG

PERIOD COVERED
July 1, 1976 through September 30, 1977

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, Laboratory of Biochemical Genetics	LBG NHLBI
OTHER:	Donald G. Puro	Research Associate	LBG NHLBI
	Phillip Nelson	Chief, Behavioral Biology Branch	BB NICHD
	Clifford Christian	Special Fellow	BB NICHD
	Steven Wilson	Guest Worker	LBG NHLBI
	Haruhiro Higashida	Visiting Fellow	LBG NHLBI
	Carolyn B. Smith	Staff Fellow	LBG NHLBI

COOPERATING UNITS (if any)

Behavioral Biology Branch, NICHD

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.2	5.0	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)

Long range objectives are to define reactions which are required for synapse formation or for regulation of transmission of information across the synapse.

1087

Project Description:

To understand the molecular nature of the factors that determine the specificity of synapse formation it is experimentally advantageous to have homogenous populations of neurons that form synapses. Since normal neurons do not divide, clonal lines of neuroblastoma cells and somatic cell hybrids derived from neuroblastoma cells were generated and characterized with respect to components known to be required for transynaptic communication such as neurotransmitters, receptors and action potential ionophores. Fusion of mouse neuroblastoma cells with rat glioma cells yielded a clonal hybrid cell line NG108-15, which synthesizes, stores and excretes acetylcholine, properties which are not expressed by the parental cell lines, and which forms synapses with mouse striated muscle cells. The clonal hybrid cell line provided an opportunity to test the specificity of synapse formation for if the formation of synaptic connections between neurons and muscle cells were dependent on interactions between specific cell recognition molecules, then discrete classes of muscle cells with different specificities for synapse formation might be expected. In the past year we showed that the NG108-15 hybrid cells form synapses with cells from different muscles and from different species. Thus, most striated muscle cells constitute a single class of cells with respect to the formation of synapses. A hypothesis was proposed that part of the specificity of the normal neuromuscular synapse is acquired after the synapses form by a process of selection that reduces the number of synapses and is dependent upon transmission across the synapse.

A clonal line of mouse striated muscle cells, G8, was established which differentiates well in vitro and forms synaptic connections with NG108-15 cells. Thus two clonal cell lines can be used to study the process of synapse formation. G8 muscle cells were highly sensitive to acetylcholine at all sites tested on the surface of the myotubes, whereas primary mouse myotubes were sensitive to acetylcholine primarily at localized sites on the myotube plasma membrane. G8 muscle cells thus are defective with respect to localization of nicotinic acetylcholine receptors.

Other cell lines of neural origin were obtained which synthesize acetylcholine and adhere well to muscle cells but do not form synapses with muscle cells. Further study is needed to explore the properties of the synapse defective cell lines.

The retina is another model system which we have used in the study of the reactions involved in synapse formation. In the past year we have found that embryonic chick retina neurons which synthesize acetylcholine are able to make, then break functional synaptic connections with rat striated muscle cells. As the inappropriate retina neuron-muscle synapses are eliminated, synapses form between neurons.

Significance to Biomedical Research: The clonal cell lines can be used as model systems for biochemical studies on synapse formation, to define the properties of synapses, and to correlate biochemical events with developmental and electrophysiological phenomena.

Proposed Course: Current studies focus on determining the reactions which are required for synapse formation and factors regulating these reactions.

Publications:

1. Puro, D. G. and Nirenberg, M.: On the specificity of synapse formation. Proc. Natl. Acad. Sci. USA 73: 3544-3548, 1976.

2. Christian, C., Nelson, P., Peacock, J. and Nirenberg, M.: Synapse formation between two clonal cell lines. Science 196: 995-998, 1977.

3. Vogel, Z. and Nirenberg, M.: Localization of acetylcholine receptors during synaptogenesis in retina. Proc. Natl. Acad. Sci. USA 73: 1806-1810, 1976.

4. Vogel, Z., Daniels, M. P. and Nirenberg, M.: Synapse and acetylcholine receptor synthesis by neurons dissociated from retina. Proc. Natl. Acad. Sci. USA 73: 2370-2374, 1976.

5. Chalazonitis, A., Minna, J. D. and Nirenberg, M.: Expression and properties of acetylcholine receptors in several clones of mouse neuroblastoma x L cell somatic hybrids. Exp. Cell.Res. 105: 269-280, 1977.

6. Nelson, P. G., Christian, C. N., Daniels, M. P., Henkart, M., Bullock, P., Mullinax, D. and Nirenberg, M.: Formation of synapses between cells of a neuroblastoma glioma hybrid clone and mouse myotubes. Brain Research, in press.

7. Christian, C. N., Nelson, P. G., Bullock, P., Mullinax, D. and Nirenberg, M.: Pharmacologic responses of cells of a neuroblastóma x glioma hybrid clone and modulation of synapses between hybrid cells and mouse myotubes. Brain Research, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00011-02 LBG

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

The Development of Chick Embryo Retina

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

PI:	F. G. De Mello	Visiting Associate	LBG NHLBI
	Marshall Nirenberg	Chief, LBG	LBG NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Biochemical Genetics

SE
Section on Molecular Biology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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)

Some biochemical aspects of chick embryo retina differentiation were studied: 1) An alternate route for GABA synthesis was characterized in the retina; which depends upon the conversion of putrescine to GABA. 2) Glutamic acid decarboxylase specific activity in the retina, increased during the course of embryonic development, either when measured in ovo or in aggregate cultures. 3) The presence of GABA in the culture medium prevented the development of glutamic acid decarboxylase activity in aggregate cultures. 4) The proposed course of this project is to attempt to correlate the biochemical changes observed, with synaptogenesis in the retina.

1090

Project Description:

Objectives: The objective of the project is to study the biochemical step required for synaptogenesis in chick embryo retina.

Major Findings: Two pathways for γ-aminobutyric acid synthesis were found in chick embryo retina. The first pathway depends upon the conversion of putrescine to ornithine decarboxylase and the subsequent conversion of ornithine to γ-aminobutyric acid. The second route of synthesis is dependent upon the conversion of glutamic acid to γ-aminobutyric acid, catalyzed by glutamic acid decarboxylase. Elevation of cAMP levels in neuroblastoma cells was shown to induce ornithine decarboxylase activity. Thus, in the developing embryo, neurotransmitters which affect cAMP levels may regulate ornithine decarboxylase activity and thereby control the rate of GABA synthesis from ornithine.

GABA was found to regulate the specific activity of glutamic acid decarboxylase in cells dissociated from chick embryo retina and cultured in vitro.

Significance to Biomedical Research: These findings show that GABA can be synthesized by a novel pathway and provide new insight on the relationship and regulation of GABA synthesis.

Proposed Course: To determine the effect of retina neurotransmitters and other compounds on retina synaptogenesis.

Publications:

1. De Mello, F. G., Bachrach, U. and Nirenberg, M.: Ornithine and glutamic acid decarboxylase activities in the developing retina. J. Neurochem. 27: 847-851, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00012-03 LBG

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Muscarinic Acetylcholine Receptors of Cultured Cell Lines

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:	Orest Hurko	Staff Associate	LBG NHLBI
	William Klein	Guest Worker	LBG NHLBI
	Wolfgang Burgermeister	Guest Worker	LC NIAMDD
	Bernhard Witkop	Chief, LC	LC NIAMDD

COOPERATING UNITS (if any)

Laboratory of Chemistry, NIAMDD

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NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Studies on muscarinic acetylcholine receptors address the following questions: (1) How does ligand activation of the receptor influence the behavior of other membrane bound proteins? (2) What is the mechanism of activation and desensitization of the receptors of activation, and (3) What are the properties of the different forms of the receptor.

The approach taken is the study of both ligand-binding and physical properties of muscarinic receptors, comparing particulate and solubilized forms of the receptors.

1092

PHS-6040
(Rev. 10-73)

Project Description:

[^{3}H]-Quinuclidinyl-benzilate (QNB) and [^{3}H]-scopolamine were used to study excitatory acetylcholine receptors of neuroblastoma x glioma NG108-15 cells and inhibitory acetylcholine receptors of clonal neuroblastoma N1E-115 cells. Both ligands bind with high affinity to muscarinic acetylcholine receptors of the cells. The apparent dissociation constants are 0.1 nM (NG108-15) and 0.06 nM (N1E-115) for [^{3}H]QNB; and 0.5 nM (NG108-15) and 0.4 nM (N1E-115) for [^{3}H]-scopolamine.

If each muscarinic acetylcholine receptor molecule contains one binding site for QNB, then the average NG108-15 or N1E-115 cell possesses 30,000 and 22,500 acetylcholine receptors/cell, respectively. Activation of inhibitory acetylcholine receptor of N1E-115 cells results in the synthesis of approximately 20,000 molecules of cGMP in 15-20 sec (670 cGMP molecules synthesized/sec/receptor), cell hyperpolarization (usually -15 mV for 10-15 sec), and desensitization of the receptors; whereas, activation of one excitatory muscarinic acetylcholine receptor of NG108-15 cells results in the synthesis of <1 molecule of cGMP/receptor/sec, depolarizes the cell and desensitizes the receptor within 30 sec with respect to cGMP accumulation and ionophore activity. The results suggests that the desensitized form of the receptor concomitantly converts the receptor to a form which is a potent inhibitor of adenylate cyclase. The receptor mediated inhibition of adenylate cyclase is dependent on a ligand which activates receptors, is blocked by receptor antagonists, but is not desensitized by receptor activators. Hill coefficients of approximately 1 were found for receptor antagonists and approximately 0.5 for receptor activators. Antagonist binding therefore can be described as interaction of ligand with an independent, noninteracting class of receptors whereas binding of receptor activators exhibits either negative cooperativity or heterogeneity of binding sites. [Ligand·Receptor] heterogeneity was demonstrated by analysis of the kinetics of [^{3}H]-QNB association with and dissociation from the receptors revealed 3 sequential reactions which affect the properties of the [carbamylcholine·receptor] complexes formed. The functional coupling of the [activator·receptor] complex with the adenylate cyclase complex provides further evidence for multiple forms of [ligand·receptor].

The receptors from both cell lines were solubilized from membranes and 2 methods of assaying the solubilized receptors were devised. The apparent negative cooperativity of the [activator·receptor] complex was abolished by solubilization. A sequence of reactions was proposed concerning the mechanism of receptor activation, desensitization and the actions of receptors based on the different forms of [ligand·receptor] complex and the reactions which were found.

Proposed Course: Further purification and characterization of receptors is needed to define the properties of excitatory and inhibitory acetylcholine receptors and the mechanisms of coupling the receptors to elevation of intracellular cAMP levels or inhibition of adenylate cyclase.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00013-03 LBG

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Developmental Regulation of Excitability

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

PI: William A. Catterall Research Chemist LBG NHLBI

COOPERATING UNITS (if any)

Dr. Jonas Galper, Cardiovascular Division, Peter Bent Brigham Hospital

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NIH, NHLBI, Bethesda, Maryland 20014

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 objective of this project is to define the role of receptors and action potential ionophores in the development of excitable tissue. Our current efforts are directed toward understanding the role of electrical stimuli, cell-cell interaction, and nerve-muscle interaction in the developmental changes in receptors and action potential ionophores in heart and skeletal muscle.

1094

Project Description:

Objectives: The objectives of this project are (1) to devise biochemically manipulable cell culture systems for studying the development of action potential generation and receptor function in excitable cells, (2) to use these cell culture systems to document the changes in action potential generation and receptor function occurring during development, and (3) to understand the role of hormonal stimuli, cell-cell interaction, and synapse formation in these developmental changes.

Methods employed: Muscarinic acetylcholine receptors were detected by binding of ^{3}H-quinuclidinyl benzilate (QNB) prepared as described in project report no. Z01 HL 00012-02 LBG.

Major findings: Further studies were made of the muscarinic acetylcholine receptors in unresponsive embryonic chick hearts (day 3 in ovo) and responsive chick hearts (day 9 in ovo). Extensive analysis of ligand binding by active and inactive receptors revealed that a small time-dependent increase in the affinity of carbamylcholine for the receptor was the only difference observed. Thus, the physiologically inactive receptors in young embryonic hearts must be defective in some aspect of receptor function subsequent to ligand binding.

Kinetic analysis of QNB binding in heart was undertaken in view of the previous observation (project report no. Z01 HL 00012-02 LBG) that binding occurs in a two step reaction to receptors of brain and neuroblastoma and hybrid cells. We confirm that, in heart, QNB first forms a rapidly reversible complex with the muscarinic receptor followed by a more slowly reversible complex. Kinetic data conclusively show that this is a two step sequential reaction. It is likely that it represents a QNB induced conformational change in the receptor. This conformational change occurs in both active and inactive receptors.

Significance to biomedical research: These results provide new insight into the developmental regulation of membrane macromolecules involved in information transfer and processing in the nervous system and in maintenance of normal beating in heart.

Proposed course: This project will be terminated because the principal investigator is leaving NIH.

Publications:

1. Galper, J. B., Klein, W. and Catterall, W. A.: Muscarinic acetylcholine receptors in developing chick heart. J. Biol. Chem., in press, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00014-02 LBG

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Storage and Release of Molecules Required for Synaptic Communication

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
	Richard McGee	Pharmacology Research Associate	LBG NHLBI
	Steven Wilson	Guest Worker	LBG NHLBI

COOPERATING UNITS (if any)

National Institute of General Medical Sciences

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NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.25	2.25	

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 objectives are to define the steps which are required for neurotransmitter storage and release from neuroblastoma and hybrid cell lines and factors which regulate these reactions.

1096

Project Description:

Major Findings: Methods were devised for measuring fmol quantities of [^{3}H]-acetylcholine released by NG108-15 cells. Serotonin or $PGF_2\alpha$ which depolarize NG108-15 cells were found to stimulate acetylcholine release from the cells; whereas PGE_1 had little or no effect. Activation of the Na^+ action potential ionophore with veratridine or the addition of KCl also evoked acetylcholine release from cells. The ability of cells to store and release acetylcholine was found to be regulated. Methods were found for shifting populations of cells from a competent state with respect to the storage and release of acetylcholine to a defective state and vice versa. In consequence synapse formation by clonal cells can be regulated.

Significance to Biomedical Research: A model system has been established which will be used to study the reactions which are required for acetylcholine storage and release and factors which regulate these reactions.

Proposed Course: The directions to be pursued are the further evaluation of the responses of the cells to various pharmacological agents, defining the mechanisms of acetylcholine storage and release and the regulation of these processes.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00015-02 LBG

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Regulation of adenylate cyclase by alpha-adrenergic receptors

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:	Steven L. Sabol	Research Associate	LBG NHLBI
	Saburo Ayukawa	Visiting Associate	LBG NHLBI

COOPERATING UNITS (if any)

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NHLBI, NIH, Bethesda, Maryland 20014

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)

The role of the cyclic nucleotides adenosine 3':5' monophosphate (cyclic AMP) and guanosine 3':5' monophosphate (cyclic GMP) in synaptic transmission is under study using cultured cells of neural origin. The topics of interest during the current year have been the following: 1) The alpha-receptor-mediated inhibition of adenylate cyclase activity by norepinephrine in neuroblastoma x glioma hybrid cells; and 2) characterization of a compensatory increase of adenylate cyclase activity in cells treated for 10 hours or longer with norepinephrine and study of the mechanism of this increase, which results in cell tolerance to and dependence upon norepinephrine with respect to cyclic AMP synthesis.

1098

Project Description:

Objectives: Alpha-receptor activators such as norepinephrine rapidly lower cAMP levels of NG108-15 cells by inhibiting adenylate cyclase activity. Furthermore, prolonged exposure of cells to alpha receptor agonists results in an increase in adenylate cyclase activity which compensates for the inhibition. Similar rapid inhibitions and compensatory increases elicited by opiate and muscarinic cholinergic receptor agonists have been recently observed by others. During the past year, attempts have been made to characterize these phenomena further and to elucidate the regulatory mechanisms.

Major Findings: NG108-15 hybrid cells possess α-adrengeric receptors which in concert with receptor activators inhibit adenylate cyclase. Cells were cultured in the presence of norepinephrine for 0-48 hours, then the effects of withdrawal of norepinephrine either by replacing the medium or by the addition of a receptor antagonist was tested. Withdrawal of norepinephrine resulted in a 9-fold increase in cAMP levels of intact cells. Adenylate cyclase activity also increased but to a lesser extent. Studies on the specificity of receptor antagonists showed that both the inhibition of adenylate cyclase by norepinephrine and the subsequent increase in adenylate cyclase activity are mediated by α-receptors. These and other results show that dual regulation of adenylate cyclase is a general phenomenon and that cells can become dependent upon norepinephrine, acetylcholine, or opiates. The cells develop an apparent tolerance to these compounds but in fact remain sensitive to the compound used.

NG108-15 α-receptors were characterized by studying the specific binding of [^{3}H]-dihydroergocryptine and other ligands to the receptors. The specificity of the binding sites for ligands resembles that of α-receptors. The binding of the ligand to the membrane preparation is a saturable process. The average NG108-15 cell possesses 60,000 α-receptors.

Significance to Biomedical Research: 1. The fact that dual regulation of NG108-15 adenylate cyclase has been observed now with three classes of inhibitors each mediated by a different species of receptor, suggests that dual regulation may be a general phenomenon. 2. Norepinephrine released at adrenergic synapses may regulate cAMP levels in post-synaptic or pre-synaptic cells by the mechanism discussed here. Such regulation may modulate the cell's responsiveness to ligands for other species of receptors which activate adenylate cyclase and thus may affect information transfer in the nervous system.

Proposed Course: The potencies of α-receptor activators and antagonists with respect to inhibition of adenylate cyclase will be compared with the effects of ligand binding to NG108-15 alpha-receptors. The mechanism of coupling inhibition of adenylate cyclase with a subsequent compensatory increase in enzyme activity will be studied further.

Effects of α-adrenergic activators and antagonists on [^{3}H] dihydroergocryptine binding will be determined to define the specificity of the α-receptor and the kinetics of binding. The regulation of receptor concentration will be studied.

Publications:

1. Archer, Ellen G., Breakefield, Xandra O. and Sharata, Mary N.: Transport of tyrosine, phenylalanine, trytophan and glycine in neuroblastoma clones. J. Neurochem. 28: 127-135, 1977.

1100

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00016-02 LBG

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Acetylcholine Receptor-Mediated Regulation of Adenylate Cyclase in Hybrid Cells

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
	Neil M. Nathanson	Guest Worker	LBG NHLBI

COOPERATING UNITS (if any)

Postdoctoral Fellow, Muscular Dystrophy Association

LAB/BRANCH

Laboratory of Biochemical Genetics

Section on Molecular Biology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

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)

The results show that cells can become dependent upon and tolerant to activation of excitatory muscarinic acetylcholine receptors and suggest that neurotransmitter-receptor interactions can exert long-lived effects on macromolecules required for synaptic transmission.

1101

Project Description:

Objectives: To study muscarinic acetylcholine receptor mediated regulation of adenylate cyclase activity.

Major Findings: NG108-15 hybrid cells possess excitatory muscarinic acetylcholine receptors which also are coupled to the inhibition of adenylate cyclase. Cells were cultured in the presence of carbamylcholine for 0-48 hours, then the effects of withdrawal of carbamylcholine was tested. Withdrawal of carbamylcholine resulted in a 5-fold increase in cAMP levels of intact cells. Atropine (1 μM) blocked both the carbamylcholine dependent inhibition of adenylate cyclase and the delayed increase in adenylate cyclase activity. Adenylate cyclase activity also increased but to a lesser extent. These results show that NG108-15 cells can become dependent upon and tolerant to acetylcholine as well as opiates.

Significance to Biomedical Research: The results show that cells can become dependent upon and tolerant to activation of excitatory muscarinic acetylcholine receptors and suggest that neurotransmitter-receptor interactions can exert long-lived effects on macromolecules required for synaptic transmission.

Proposed Course: Work on this project will be incorporated into other projects in the coming year.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00017-02 LBG

PERIOD COVERED

July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Acetylcholine Receptors in the Developing Nervous System

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

PI:	Hiroyuki Sugiyama	Visiting Associate	LBG NHLBI
	Marshall Nirenberg	Chief, LBG	LBG NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Biochemical Genetics

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Section on Molecular Biology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	1.3	0.3

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The goal of this project is to define the properties of muscarinic and nicotinic acetylcholine receptors and acetylcholinesterase during development of chick embryo retina. Thus far we have elucidated (1) the specificity and affinities of muscarinic and nicotinic acetylcholine receptors for receptor activation and antagonists, (2) the number of receptors were defined as a function of developmental age of the retina, and (3) the location of nicotinic and muscarinic receptors within the retina was determined.

1103

Project Description:

Objectives: The objectives are to define the biochemical properties of acetylcholine receptors before and after synaptogenesis in the retina.

Major Findings: Neurons dissociated from chick embryo retina and maintained *in vitro* were found to reaggregate and form, *in vitro*, approximately 1 x 10^9 synapses per mg of protein. Three types of synapses and several subtypes were identified which closely resemble those of the intact retina.

Chick embyro retina was found to be a rich source of both muscarinic and nicotinic acetylcholine receptors. Both muscarinic and nicotinic acetylcholine receptors are synthesized before synapses appear in the retina; however, during development, nicotinic acetylcholine receptors become associated predominantly with neurites in the synaptic layers of the retina. Muscarinic acetylcholine receptors also were found to localize in the inner synaptic layer of the retina, but the receptor distribution differs from that of nicotinic acetylcholine receptors. The properties of muscarinic acetylcholine receptors were determined at different developmental ages and were compared with the properties of muscarinic inhibitory and excitatory receptors of neuroblastoma and hybrid cells.

Significance to Biomedical Research: Information was obtained which serves as a basis for further studies on the role of acetylcholine receptors in synapse formation.

Proposed Course: Further studies on receptor properties are planned.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 00151-07 LBG

PERIOD COVERED
July 1, 1976 through September 30, 1977

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	LBG NHLBI
OTHER:	Jose E. Gonzalez	Staff Fellow	LBG NHLBI
	Chandan Prasad	Staff Fellow	LBG NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Biochemical Genetics

SECTION
Section on Macromolecules

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NHLBI, NIH, Bethesda, Maryland 20014

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3	2	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

It is the long-range purpose of this project to understand the biological role and mechanisms regulating the nucleotides, cyclic AMP and cyclic GMP in Escherichia coli. The focus of interest during the current year was to study the mechanism by which a sugar transport system known as phosphoenolpyruvate: glucose phosphotransferase system regulates the activity of adenylate cyclase. We found that the membrane-bound sugar recognition proteins of this transport system are essential for adenylate cyclase regulation by glucose but do not influence the activity of the enzyme in the absence of glucose.

1105

Project Description:

Major Findings: This project is aimed at elucidating the mechanism by which the enzyme adenylate cyclase of Escherichia coli is regulated. Our previous studies indicated that this enzyme, which synthesizes the nucleotide cyclic AMP, can be inhibited by glucose. The nature of the inhibition by glucose is complex, since glucose has no demonstrable effect on the enzyme in cell-free extracts, while it does inhibit the enzyme activity in whole cells. The development of a permeabilized cell preparation that has high adenylate cyclase activity which can be inhibited by glucose has allowed us to accumulate substantial information about the mechanism for regulation of this enzyme. The focus of our recent attention has been an investigation of the interaction of the adenylate cyclase enzyme with the components of the sugar transport system known as the phosphoenolpyruvate:glucose phosphotransferase system or PTS. The PTS is represented by the following scheme:

1) Phosphoenolpyruvate (PEP) + Enzyme I $\longrightarrow$ Enzyme-I-P + pyruvate

2) Enzyme-I-P + HPr $\longrightarrow$ HPr-P + Enzyme-I

3) HPr-P + sugar $\xrightarrow{\text{Enzyme II}}$ sugar-phosphate + HPr

Our previous studies involved an examination of the role of the proteins Enzyme I and HPr in the adenylate cyclase regulation mechanism. These two proteins are found in the soluble fraction of the cell and are required for the transport of all "PTS" sugars. We found, by studying the properties of mutant cells, that the HPr protein was dispensable while the Enzyme I protein was essential. These and other data have led us to formulate the model that the critical feature of the adenylate cyclase regulation mechanism involves the state of phosphorylation of the Enzyme I protein.

This past year we have continued our studies in the direction of a further elucidation of the mechanism of adenylate cyclase regulation. A part of this study involved the use of glucose analogues as inhibitors of adenylate cyclase. We found that those analogues with variations of the substituents about carbon atoms 1 or 2 (e.g. α-methylglucoside or 2-deoxyglucose) are inhibitory and they are also substrates of the phosphoenolpyruvate-dependent sugar phosphotransferase system. Analogs with changes in other parts of the molecule (e.g. 3-O-methylglucose or galactose), L-glucose and several disaccharides and pentoses, do not inhibit adenylate cyclase and are not substrates of the transport system. This correlation provides further evidence for a functional relationship between the adenylate cyclase and phosphotransferase systems.

A further study was carried out with mutants defective in the glucose enzymes II of the phosphotransferase system (designated GPT and MPT); these two activities are measured by phosphorylation of α-methylglucoside and 2-deoxyglucose, respectively. The wild-type parent phosphorylates both analogues and both inhibit adenylate cyclase. In the GPT^- mutant, α-methylglucoside does not inhibit adenylate cyclase and is not phosphorylated, while 2-deoxyglucose is inhibitory and phosphorylated. In the GPT^-MPT^- double mutant, adenylate cyclase activity is present, but neither α-methylglucoside

nor 2-deoxyglucose inhibits adenylate cyclase and neither sugar is phosphorylated. These studies demonstrate that glucose inhibition of adenylate cyclase in toluene-treated cells requires an interaction of this sugar with either the GPT or MPT enzyme II of the phosphotransferase system.

Proposed Course: We plan to continue our study of the mechanism of interaction of adenylate cyclase with the sugar transport system. A further study of the properties of transport deficient mutants should help our understanding of this system.

Publications:

1. Harwood, J. P. and Peterkofsky, A.: Regulation of Escherichia coli adenylate cyclase by glucose: A model for the study of receptor-coupled adenylate cyclase systems. In: Beers, R. F., Jr. and Bassett, E. G. (Eds.): Receptors for Viruses, Antigens and Antibodies, Polypeptides Hormones and Small Molecules. Proc. Ninth Miles Int. Symp., Baltimore, June 4-6, 1975. New York, Raven Press, pp. 393-410, 1976.

2. Harwood, J. P., Gazdar, C., Prasad, C., Peterkofsky, A., Curtis, S. J., and Epstein, W.: Involvement of the glucose enzymes II of the sugar phosphotransferase system in the regulation of adenylate cyclase by glucose in Escherichia coli. J. Biol. Chem. 251: 2462-2468, 1976.

3. Peterkofsky, A.: Cyclic Nucleotides in Bacteria. In: Greengard, P. and Robison, G. A. (Eds.): Advances in Cyclic Nucleotide Research. Raven Press, New York, Vol. 7, pp. 1-48, 1976.

4. Peterkofsky, A.: Regulation of Escherichia coli adenylate cyclase by Phosphorylation-Dephosphorylation. Trends in Biochemical Sciences 2: 12-14, 1977.

5. Peterkofsky, A.: The Mechanism of Regulation of Escherichia coli Adenylate Cyclase. In: Abou-Sabé, M. (Ed.): Cyclic Nucleotides and the Regulation of Cell Growth. Dowden, Hutchinson and Ross, Stroudsburg, Pa., pp. 27-35, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 HL 00152-04 LBG

PERIOD COVERED
July 1, 1976 through September 30, 1977

TITLE OF PROJECT (80 characters or less)

Mechanisms in Protein Synthesis

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

PI:	Alan Peterkofsky	Research Chemist	LBG NHLBI
OTHER:	Chandan Prasad	Staff Fellow	LBG NHLBI
	Takashi Matsui	Visiting Fellow	LBG NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Biochemical Genetics

SECTION
Section on Macromolecules

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NHLBI, NIH, Bethesda, Maryland 20014

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2	2	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

It is the goal of this project to continue our studies in the area of protein and peptide synthesis and function. Our focus of interest in the past year was two-fold. Firstly, we examined the mechanism for initiation of the synthesis of chicken ovalbumin and found evidence that the protein was initiated by methionine. Secondly, we studied the nature of metabolism of thyrotropin releasing hormone. We found that hamster hypothalamic extracts contain two different enzymes that act on thyrotropin releasing hormone. One enzyme is a pyroglutamyl peptidase and the other enzyme is an amidase.

1108

Project Description:

Major Findings:

Synthesis of Ovalbumin. Ovalbumin is a protein that in its natural form, contains N-acetylglycine at its amino-terminus. We have been interested in elucidating the mechanism by which proteins containing blocked amino-termini are synthesized. In the present study, we examined the incorporation of [^{35}S]-methionine into ovalbumin in chicken oviduct magnum cells. The purification of [^{35}S]-methionine-labelled ovalbumin from total oviduct proteins was accomplished by dialysis of a crude extract at pH 3.6 followed by chromatography on carboxymethyl cellulose. The radioactive ovalbumin eluted from the column in three peaks, containing 0, 1, and 2 moles of phosphate, respectively, per mole of ovalbumin. The kinetics of labelling in these peaks showed that the ratio of radioactivity in NH_2-terminal methionine to total incorporation was greater at 2 min of labelling than at later times. The transient labeling of ovalbumin with methionine indicates that methionine is the initiator amino acid for the synthesis of this protein, which in its native form contains amino-terminal N-acetylglycine.

Metabolism of Thyrotropin Releasing Hormone. In the present study, we examined the pathway for metabolism of thyrotropin releasing hormone in hamster hypothalamic extracts. Using a radioimmunoassay method for thyrotropin releasing hormone, the presence of thyrotropin releasing hormone metabolizing activity in various hamster tissues was demonstrated. While there was substantial activity degrading thyrotropin releasing hormone in hypothalamus, there was a notable absence of such activity in pituitary. The enzymatic activity in the hypothalamus was shown to be soluble and separable into two fractions. Analysis of the metabolic products formed by the two enzymes indicated that one possessed an amidase activity which converts thyrotropin releasing hormone to pyroglutamyl-histidyl-proline. The other fraction contains pyroglutamyl peptidase activity which converts thyrotropin releasing hormone to histidyl-proline amide. Other peptides containing amino-terminal pyroglutamic acid or carboxyl-terminal amide groups did not block the hydrolysis of thyrotropin releasing hormone, suggesting that the enzymes were specific. Some inhibitors preferentially blocked the activity of one or the other enzyme. Of possible biological significance is the observation that thyroid-stimulating hormone inhibited the amidase activity while hydrocortisone inhibited the pyroglutamyl-peptidase activity.

Proposed Course: The metabolic product of pyroglutamyl-peptidase action on thyrotropin releasing hormone is histidyl-proline amide. This compound has not previously been implicated as a metabolite of thyrotropin releasing hormone. We plan to further examine the metabolism and biological properties of this compound.

Publications:

1. Prasad, C. and Peterkofsky, A.: Initiation of Ovalbumin Synthesis in Chicken Oviduct Magnum: Transient Labelling of the NH_2-terminus by Methionine. Arch. Biochem. Biophys. 175: 730-736, 1976.

2. Prasad, C. and Peterkofsky, A.: Demonstration of Pyroglutamyl-peptidase and amidase activities toward thyrotropin-releasing hormone in hamster hypothalamus extract. J. Biol. Chem. 251: 3229-3234, 1976.

Annual Report of the
Pulmonary Branch
National Heart, Lung, and Blood Institute
July 1, 1976 through September 30, 1977

The major function of the lung is to exchange gases between the atmosphere and the blood. To accomplish this, the lung has evolved as a complex structure which brings together the atmosphere and the blood in a fashion which is finally regulated to insure maximum efficiency. The major thesis of this laboratory is that the process of gas exchange is critically dependent on lung cells in their ability to maintain lung structure and function. The adult lung consists of approximately 40 cell types and a complex extracellular matrix composed of collagen, elastic fibers, and the ground substance. These connective tissue components provide a scaffolding to define lung structure and are critical determinants of the mechanical properties of lung during the processes of gas exchange. Disturbances of the connective tissue of the lung, such as that found in emphysema or the fibrotic lung disorders, has profound effects on the ability of lung to function as an efficient organ of gas exchange. As a comprehensive approach to understanding the composition and regulation of lung connective tissue in health and disease, the Pulmonary Branch utilizes several approaches. Most important of these are the isolation and characterization of lung cells that can be studied in culture. This approach has enabled us to carefully define the composition, synthesis, and degradation of the major connective tissue components of lung with particular emphasis upon collagen. We also have spent a significant effort into understanding the connective tissue of the intact animal and human in both the normal condition and in the disease state. The following is a summary of the ongoing projects in the Pulmonary Branch for the past year.

I. Isolation, Culture, and Characterization of Lung Cells

The use of biochemical technology to understand human lung disease is limited by the complexity of the organ, the unavailability of large quantities of lung cells, and the multitude of variables involved in attempting to study the lung *in situ*. Even though the biochemist can approach the study of lung cells with a vast armamentarium of concepts and sophisticated methodologies, the inherent complexity of dealing with multiple cell types immediately hinders the sorting out of the contribution of each cell type to the biochemical parameters being measured. In addition to the problems of cell purity, the study of lung cells *in situ* is compounded by the multitude of factors which impinge on each cell type to attempt to vary its inherent properties. Thus, to reduce the number of (mostly unknown) variables to a minimum, it has become necessary to develop techniques by which individual populations of lung cells can be isolated, cultured, and studied. Using a variety of physical, enzymatic, and cloning methods, we have been able to isolate and/or culture the following cell types: (1) alveolar macrophages from the adult human, adult rabbit, and adult cat; (2) fibroblasts from normal adult human lung and from lung of patients with a variety of pulmonary disorders, fetal human lung, newborn rabbit lung, fetal rabbit lung, newborn rat lung, and fetal cat lung; (3) epithelial cells from fetal cat lung; and (4) alveolar Type II cells from the adult rabbit lung. By evaluating the type of proteins secreted by these cells, it is possible to characterize these cells in culture as distinct cell types

1111

with characteristic differentiated properties. These methods have been utilized to demonstrate that human fetal fibroblasts maintain the same differentiated state in culture over greater then 40 population doublings. In a detailed study of the ontogeny of fibroblasts, we have found that while fetal lung and fetal skin fibroblasts are almost identical, the adult lung and the adult skin fibroblasts show subtle but specific differences in their differentiated characteristics.

II. Composition of the Extracellular Matrix

Collagen is the most abundant protein in lung comprising 10 to 20% of the adult lung by dry weight. The lung parenchyma of animal and human lung is composed of at least two types of collagen: Type I collagen $[\alpha 1(I)_2 \alpha 2]$ and Type III collagen $[(\alpha 1)III)]_3$. In addition, it is likely that basement membrane collagen is present in lung parenchyma as well. Although it is not clear whether this material is composed of a single form of collagen or a multiple species, basement collagen probably represents less than 10% of the total interstitial collagen. The interest in collagens Type I and III revolve primarily around the general concept that they have different morphological, and probably mechanical, properties. Type I collagen is the thick, cross-banded fibrils that are stiff and noncompliant. In comparison, Type III collagen is the loose, randomly arrayed fibrils that used to be called reticulin. Although the evidence is indirect, it is likely that Type III collagen is more compliant than Type I. Thus, the relative amounts of Type I and Type III collagen within the alveolar interstitium probably have profound effects on the mechanical properties of the lung. The purpose of these studies are to establish the quantities of Type I and Type III collagen in lung parenchyma in both health and disease. Two approaches are being used: (1) evaluation in the changes in the ratio of Type I and Type III collagens with lung growth and development; and (2) evaluation in the changes of parenchymal lung collagen Types I and III in health and disease. In the latter we have concentrated primarily on evaluating the fibrotic lung disorders.

III. Control of the Synthesis and Degradation of the Extracellular Matrix

The maintenance of the extracellular matrix of lung is the responsibility of the lung cells within and bordering the alveolar interstitium. The types, quantities, and locations of the connective tissue constituents that these cells synthesize and degrade are dependent on the inherent differentiated properties of these cells as modulated by cell-cell interactions as well as hormonal, immune and inflammatory processes.

Studies of the specific collagen types synthesized by cells of the lung have shown that AK-D, a tissue culture analog of the feline fetal epithelial cell (probably a Type I cell or a cell in transition from the Type II to Type I) synthesizes both Type I and Type III collagen. HFL-1, a human fetal lung fibroblast, also synthesizes Type I and Type III collagen. Studies on the maintenance of the differentiated state with regard to the relative rigidity of the synthesis of Types I and III cells in culture have shown that NB-6, a newborn rabbit lung fibroblast, synthesizes approximately 65% Type I and 35% Type III. Evaluation of the relative amounts of these collagens synthesized

at different times after subcultivation and at different subcultivation numbers have demonstrated that the same amount of collagen Types I and III are synthesized by the cell at all times. Detailed studies of the rates of collagen synthesis of HFL-1, a human fetal lung fibroblast, have demonstrated that it synthesizes the same relative amounts of collagen over many population doublings. However, there appears to be a significant difference in the relative amount of collagen synthesized by these cells as to whether they are in the log or confluent phase of cell growth. Preliminary studies of cell-mediated immune control on the rates of collagen synthesis by lung cells suggest that lymphocytes produce materials which selectively reduce the rates of collagen synthesis by lung cells. Studies of hormonal controls of collagen synthesis have demonstrated that PGE_1, choleratoxin and isoproterenol all cause elevations in intracellular cyclic AMP with an associated selective decrease in rates of collagen synthesis by these cells.

Evaluation of the rates of collagen synthesis by explants of human lung from patients with idiopathic pulmonary fibrosis have demonstrated that these patients' lung cells synthesize the same amounts of collagen per cell as does the normal. This finding suggests that the disorder is not due to lung cells synthesizing excess amounts of collagen, but rather associated with lung cells synthesizing normal amounts in the wrong places.

The alveolar macrophage secretes a collagenase that is in a latent, inactive form. This same cell secretes a neutral protease which will activate this collagenase. In the process of activation, the molecular weight of the collagenase is reduced from approximately 40,000 to 30,000 daltons. Likewise, fetal lung fibroblast collagenase is also in a latent form. While the alveolar macrophage and human fibroblast collagenase will degrade collagen Types I and III at equal rates, human neutrophil collagenase will degrade Type I collagen at a rate 15 times that at which it attacks Type III collagen. Detailed studies of lung explants have demonstrated that 30 to 40% of the newly synthesized collagen is degraded intracellularly. This occurs only during a very short time during the period of synthesis and secretion of the collagen molecule. Although the processes which control this intracellular degradation are not clear, it may be a way in which the cell regulates the quality of the collagen which it secretes.

IV. Control of Cell-Cell and Cell-Matrix Interactions

The major cell surface protein of cultured fibroblasts is a large (220,000 dalton) glycoprotein termed CSP. There is a growing body of evidence that CSP is one of the biological macromolecules responsible for orderly cell-cell and cell-substratum interactions. Current evidence suggests that CSP interacts with the extracellular matrix by associating with a specific site on the collagen molecule. The purpose of our studies in the past year have been to: (1) determine the processes by which CSP leaves the surface of normal fibroblasts; and (2) to evaluate ways in which the inflammatory system may interact with CSP to disrupt cell-cell and cell-substratum interactions. With time, human lung fibroblast CSP is progressively lost from the cell surface with a half-life of approximately 25 hours. Simultaneous with the loss of CSP from the cell surface, a similar macromolecule appears in the culture medium. Importantly, this shedding process appears to be

stoichiometric; that is, there is a one-to-one relationship between the loss of CSP from the cell surface and the appearance of a similar 220,000 dalton glycoprotein in the culture media. The 220,000 dalton media glycoprotein of normal human cultured fibroblasts is probably the culture analog of the intermediate step of the shedding of this protein from the cell surface and its eventual release into the circulation. Studies with human neutrophil proteases have demonstrated that human fibroblast CSP is remarkably sensitive to proteolysis. If small amounts of human neutrophil proteases are placed on human lung fibroblasts in culture, the cells rapidly lose their CSP on their surfaces as demonstrated by immunofluorescence. In addition, when a purified preparation of CSP is briefly exposed to human neutrophil proteases, the preparation loses its ability to agglutinate formalin treated sheep red blood cells and to attach Chinese hamster ovary cells to a collagen substrate. These studies suggest that one of the effects of the inflammatory process is to destroy macromolecules such as CSP so that cellular order is disrupted. This has major implications in relation to inflammatory disorders, not only in lung, but other organs as well.

V. Clinical Studies of Fibrotic Lung Disease

The Pulmonary Branch has undertaken a detailed study of patients with pulmonary fibrosis, particularly those with environmental etiologies and those with idiopathic pulmonary fibrosis (IPF). The latter represents a chronic, devastating illness, invariably resulting in death an average of 4 to 5 years from the onset of symptoms. Studies in the past year of patients with idiopathic pulmonary fibrosis have demonstrated the following:

(1) A significant number of patients with this disease demonstrate abnormalities in frequency dependency of compliance, flow-volume curves, and maximum flow-static recoil curves. These abnormalities correlate with the morphological assessment of small airways of patients with this disease. This data demonstrates that part of the pathogenesis of IPF is in the small airways, as well as in the pulmonary parenchyma. It gives a rational for part of the hypoxemia of the disease as being related to ventilation-perfusion abnormalities secondary to airflow problems, rather than derangement of the alveolus alone.

(2) It has been known for some time that patients with idiopathic pulmonary fibrosis reduce their arterial PO_2 with exercise. Correlative studies of the morphologic assessment of fibrosis in lung biopsies from these patients with quantitative assessment of the drop in the arterial PO_2 per unit of work done, demonstrates a striking correlation. In comparison, the conventional monitors of pulmonary function for these patients (total lung capacity, vital capacity, diffusing capacity) do not correlate with the degree of fibrosis in the open lung biopsies at all. In addition, detailed analysis of the static deflation volume-pressure curves of patients with idiopathic pulmonary fibrosis show that several derived parameters from this curve also correlate with the degree of fibrosis on open lung biopsy. With these tools, the clinician now has a means to assess the amount of fibrosis that these patients have.

(3) A significant number of patients with idiopathic pulmonary fibrosis in

mid-course develop pulmonary hypertension. Studies of the pulmonary circulation of these patients compared to a variety of pulmonary function parameters and histological evaluation of the pulmonary arteries have demonstrated: (a) all patients with diffusing capacity of less than 45% predicted have pulmonary hypertension; (b) total lung capacity and vital capacity have no correlation with the degree of pulmonary hypertension; (c) in general, as the arterial PO_2 falls, the pulmonary artery pressure rises, but it is not a direct correlation; (d) the degree of pulmonary hypertension in these patients can be predicted by emperic equations relating pulmonary function parameters; (e) there is a correlation between the number of pulmonary vessels narrowed in the open lung biopsy and general level of pulmonary hypertension; and (f) in addition to pulmonary hypertension, some patients with idiopathic pulmonary fibrosis seem to have right ventricular dysfunction as evidenced by a decreased cardiac output and elevated right ventricular end-diastolic pressure when the mean pulmonary pressure is greater than 35.

(4) ^{67}Gallium citrate scanning in patients with idiopathic pulmonary fibrosis have demonstrated that 70% of these patients will accumulate this isotope in their lung parenchyma four to eight hours after injection. A method has been developed (termed the ^{67}Gallium index) by which the uptake of gallium in these patients can be quantitated in terms of the area involved, the intensity of the uptake, and the texture of uptake. Correlation of this numerical data with the degree of cellularity on open lung biopsy and the analysis of cells recoverd by bronchoalveolar lavage demonstrated that the gallium scan is an accurate predictor of the degree of the parenchymal inflammation in these patients.

(5) Scintigraphic studies with macroaggregated albumin and ^{127}Xenon have demonstrated that patients with idiopathic pulmonary fibrosis have marked abnormalities in both ventilation and perfusion. These studies have been correlated using mathematical and computer methods to produce ventilation-perfusion ratios for each of 1,500 to 2,000 small regions of lung. It is now possible to evaluate which areas of lung are functioning normally and which areas are contributing to the patient's hypoxemia. In addition, these studies have been correlated with data obtained at right heart catheterization to demonstrate that with central venous blood gases and the ventilation-perfusion scan we can now accurately predict the arterial blood gas levels within $\pm$ 5%. Thus, it is now possible to determine the amount of oxygen being transferred in each of many small regions of lung.

(6) The fiberoptic bronchoscope has proven to be a valuable tool in the analysis of the cellular constituents of the lower respiratory tract. Studies of patients with idiopathic pulmonary fibrosis have demonstrated that they have elevated levels of neutrophils in their lower respiratory tract as well as elevated levels of IgG. Preliminary studies on the types of lymphocytes found in the lower respiratory tract have demonstrated that they have a normal ratio of T-cells and B-cells. This is in comparison to patients with chronic hypersensitivity pneumonitis (farmer's lung) that have a markedly elevated ratio of T-cells and B-cells in their lungs. Prospective studies of bronchoalveolar lavage in patients with idiopathic pulmonary fibrosis suggest that this technique may be useful for determining efficacy of anti-inflammatory therapy on the disease process. The fiberoptic bronchoscope has also been

utilized to recover the fluid bathing the epithelial surface of the lower respiratory tract of patients with idiopathic pulmonary. Analysis of enzyme constituents of this fluid have shown that while these patients have normal levels of LDH and lysozyme, they have significantly elevated levels of neutral protease and collagenase. They do not, however, have any elastase. In addition, they have significantly elevated levels of β-glucuoronidase. Since we know that the alveolar interstitium in these patients is "disordered", these findings give a rational basis for the morphological disordering.

(7) Studies of the phenotype frequencies of the A and B locus antigens of the HLA system in patients with idiopathic pulmonary fibrosis have demonstrated there is no definite statistical difference in the distribution of these antigens between idiopathic pulmonary fibrosis and healthy controls. Thus, although there are many clues that this is a disease with a genetic basis, this data suggests that the genetic determinants controlling susceptibility and/or course of the disease are either far removed from the HLA system or that the disease is one that is polygenetic.

(8) In vitro evaluation of the peripheral lymphocytes of patients with idiopathic pulmonary fibrosis has demonstrated that these cells view collagen as "non-self". Since it is the collagen that is disordered in the alveolar interstitium in this disease, this suggests that cell-mediated and immune mechanisms may play a role in the continued pathogenesis of the disease.

(9) This represents the second year of the double-blind study of the treatment of idiopathic pulmonary fibrosis with azathioprine (all patients are on cortiocosteroids with 50% on azathiprine in a blind fashion). To date, 30 patients have been enrolled in this study and it is projected that within the next year the data will approach significance such that the blind can be broken and it can be firmly established as to whether there are differences in this drug therapy for this disease.

VI. Experimental Models of Pulmonary Fibrosis

As diverse as the fibrotic lung disorders may be, they have common features which suggest that pulmonary fibrosis follows a pattern of parenchymal injury, inflammation, and cellular and extracellular matrix disordering. In most of these disorders, the factors which influence the outcome of this general pattern include the nature of the primary agent, the severity of the injury, and various genetic and acquired host factors. To evaluate the importance of the immune system in the pathogenesis of pulmonary fibrosis, we have asked whether selective deletion of parts of the immune system change host susceptibility to pulmonary fibrosis. We have begun by examining T-cell dependent cellular immunity, and have chosen the nude, athymic mouse as a model of selective deficiency of this component of the immune system. The susceptibility of this mouse to fibrosis was tested by administration of bleomycin, a drug which produces a high incidence of interstitital penumonitis and fibrosis. Bleomycin is particularly suited for a study evaluating the importance of immune mechanisms in the development of pulmonary fibrosis since it causes little, if any, immunosuppression. Bleomycin administration resulted in pulmonary lesions of widely varying severity in both the normal and nude mice. Both exhibited focal, pleural-based lesions consisting of

plural thickening, disorganization of the alveolar architecture, mononuclear interstitial infiltration and accumulation of interstitial collagen There were no differences in the susceptibility of the control mice and nude mice to bleomycin. Since the mouse lacking functioning T-cells develops pulmonary fibrosis in response to bleomycin as readily as the normal mouse, these data suggest an intact cellular immune system is not a necessary condition for the development of pulmonary fibrosis.

VII. Clinical Studies of Hereditary Lung Disease

α1-antitrypsin deficiency is a genetic disease in which those affected persons have a striking reduction of this serum glycoprotein by both functional and antigenic criteria. Normally, α1-antitrypsin is one of the principal components of the serum protease inhibitor system. In the homozygous deficiency state (phenotype zz), 80 to 90% of the affected adults have accelerated, usually fatal, obstructive pulmonary disease. Although considerable progress has been made in characterizing this protease inhibitor abnormality, clues to the precise pathophysiology and effective therapy for this disorder are lacking. Current indications suggests the zz homozygous protein is a single amino acid substitution. Most likely, this abnormality results in changes in intracellular confirmation of the molecule such that sugar sidechains cannot be added. Consequently, the altered zz protein cannot be secreted in a normal fashion by the liver cells in which it is made. Recently, the Laboratory of Clinical Investigation, NIAID, has used the drug Danazol [17α-pregnene-20-yno(2,3-d) ixoxazol 17-01] in the therapy of hereditary argioneurotic adema. Not only has this agent proved useful in the management of this clinical syndrome, it also seems to have reversed the associated biochemical defect (decreased levels of the C1 esterase inhibitor). Since the C1 esterase inhibitor is also an antiprotease made by the liver. the biochemical basis of hereditary angioneurotic edema may be very similar to that of α1-antitrypsin deficiency. For this reason, a drug trial with Danazol was started in the zz homozygous patients. These studies have shown that Danazol will increase the levels of α1-antitrypsin in the serum of these patients by 50% to 80% of the patients. Whether or not the increase from an average of 30 mg % to 45 mg % will be efficacious for the therapy of this disease or will require a great deal of further detail study. However, it is the first example of the elevation of α1-antitrypsin in these patients on a chronic basis.

Annual Report of the
Section on Molecular Pharmacology
Pulmonary Branch
National Heart, Lung, and Blood Institute
July 1, 1976 to September 30, 1977

Receptors: Dysfunctions of airway and vascular smooth muscle are most readily brought under pharmacological control by drugs acting at neuromuscular junctions, where signals are transmitted to receptors in muscle membrane by chemical agents. The characterization of receptors is expected to aid in the design of new drugs, in understanding the selectivity of certain drugs for specific organs and in understanding some diseases which appear to result from the formation of antibodies to receptor components or from inadequate receptor synthesis. Currently, the feasibility of isolating adrenergic receptors is being examined. Frozen sheep pineal bodies proved to be the most abundant of several commercial sources for β-adrenergic receptors, the latter being assayed in the usual manner by the use of agonists and antagonists to displace bound radioactive lipophilic antagonists such as L-dihydroalprenolol or L-propranolol. Pineal membranes differed somewhat from those of the nucleated erythrocytes used by other laboratories. Membranes from several species contained, in addition to β-receptors, binding sites without optical specificity but with high positive cooperativity for the binding of adrenergic antagonists. Studies with fluorescent probes showed that the phenomenon does not reflect micelle formation by alprenolol and propranolol. The supposition that the binding site might be akin to that at which propranolol exerts its local anesthetic action seems unlikely, since procaine did not unmask latent binding sites for β-adrenergic antagonists. Pineal and erythrocyte receptors appear to differ in ease of solubilization by detergents in response to sulfhydryl reducing agents and in other minor ways. Receptor concentrations make the pineal body competitive with other sources, and the qualitative differences may be resolved upon further purification.

$Na^+ + K^+$-ATPase: The cationic gradients across membranes are maintained by this enzyme, which can be isolated from rabbit kidney as a complex containing two proteins. Of these, the larger bears the catalytic site and, so long as the active complex is intact, is the only component hydrolyzed by trypsin. Since the initial attack seems to be diverted fron a point near the center to one near one end in certain sodium- and ATP-dependent conformations, electrophoretic analysis of the early products of digestion can be used as a probe for conformation. Current studies have disclosed that prior labeling of enzyme SH groups with N-ethylmaleimide (NEM) prevents the sodium- and ATP-dependent changes in trypsinolysis and increases the rate. The enzyme in the presence of Mg^{++}, ATP and K^+ assumes an enzymatically inactive conformation of unusual stability, and in this state trypsin attack on the smaller glycoprotein component becomes appreciable.

The sudden increase in potassium uptake in lymphocytes stimulated to blastogenesis by lectins would suggest an activation of the ATPase, and this has been observed in rabbit thymocytes incubated to maturation and treated with optimal concentrations of concanavalin A. Since one published report suggests that cautious removal of the small glycoprotein activates the ATPase and since con A is known to stimulate release of small glycoproteins from rabbit

thymocytes, it will be of interest to determine the role in ATPase activation of the well-known lectin-stimulated lateral movements of membrane glycoproteins. The latter movements are accompanied by enhanced fluidity of membrane lipids, and it will be necessary to determine whether fluidity changes are themselves sufficient to activate ATPase.

Application of spectroscopic methods to mechanisms of drug action: The properties of membranes from rat peritoneal mast cells, mouse mastocytoma cells and human erythrocytes and their interaction with compound 48/80 have been studied with the aid of spectroscopic probe techniques. While the interior of the mastocytoma cell membranes was found to be more fluid than that of normal rat mast cells, there was no difference in fluidity near the surface of the membranes. Compound 48/80 bound strongly to the membranes of all three cell types but did not alter membrane fluidity. Membrane-bound 48/80 is not accessible to the exterior aqueous cellular environment. Preliminary results indicate that 48/80 interacts strongly with membrane phospholipids, suggesting that the histamine-releasing properties of this compound may be due to reorganization of the lipids present in mast cells. Nuclear magnetic resonance studies of 48/80 showed that it consists of a polymer of 4-methoxy-N-methylphenylethylamine units joined by methylene bridges at the 3-position.

The effect of various factors, e.g. disease and protein concentration, on the binding of drugs to human plasma albumin has been examined. Results suggest that the reduced drug binding exhibited by albumin from uremics and neonates may be due to differing proportions of two albumin components which can be separated by isoelectric focussing. The reduction in drug binding observed when albumin concentration is increased does not appear to be due to polymerization of the protein but may result from a change in the conformation of the albumin. Electron spin resonance studies of Cu(II) binding to albumins from different species suggest that complexes are formed with the amino terminus of human and bovine albumins. No complexation was observed with canine and porcine albumins. These differences can be attributed to an absence of histidine at position 3 in the amino terminal portion of canine and porcine albumin.

The topography of the active site of horseradish peroxidase has been studied with the aid of a spin-labeled analog of benzhydroxamic acid. The electron spin resonance measurements indicate that the nitroxide moiety of this spin label becomes highly immobilized on binding to horseradish peroxidase. From the dipolar interaction between the iron atom and the spin label, the minimum distance between the two spins is approximately 14 A.

A spin-labeled analog of decamethonium has been used to monitor directly the interaction between this ligand and membrane-bound cholinergic receptor from Torpedo californica. The results indicate that the receptor shows a time-dependent increase in affinity for this antagonist. This observation may help to explain the desensitization of the neuromuscular function that is induced by prolonged contact with agonists.

Studies with anti-inflammatory drugs: It was reported earlier that many of the nonsteroidal drugs inhibited proliferation of a transformed cell line

(rat hepatoma) in culture, the order of potency of these drugs being parallel to their efficacy as anti-inflammatory agents and to their ability to inhibit prostaglandin synthetase. We have now found that a nontransformed cell line, the human fibroblast, is similarly inhibited. It would appear that these drugs have an antiproliferative activity which is relevant to their anti-inflammatory action, but since the incorporation of isotopic precursors into protein and nucleic acid was not inhibited in the presence of these drugs, they do not seem to act by direct inhibition of protein or nucleic acid synthesis.

The effect of the drugs is readily reversed by washing. After a delay of about 15 hr, logarithmic growth of the cultures is resumed at a rate identical to that of controls. Viability of the cells as measured by trypan-blue exclusion is not impaired, even after exposure to high concentration (0.4 mM) of indomethacin. The kinetics of resumption of growth and the apparent change in morphology of the cells at 5 hr suggest that the cells may have become synchronous, but this remains to be proven by DNA measurements.

The inhibition of culture growth is not reversed by the addition of the prostaglandins. The prostaglandins alone inhibit growth, although the inhibition differs from that induced by the anti-inflammatory drugs. Inhibition is apparent within 60 min and becomes irreversible after exposure for several hours. No prostaglandin synthesis could be detected in the hepatoma cultures by radioimmunoassay or by incorporation of the radioactively labeled precursor, arachidonic acid. These studies provide no indication that the anti-proliferative action of the anti-inflammatory drugs is due to inhibition of prostaglandin production.

Studies of the role of histamine in gastric secretion: The recent development of centrifugal elutriation for separation of the different cell populations of the gastric mucosa by Morton Grossman and associates at the Center for Ulcer Research and Education at the Wadsworth V. A. Hospital, Los Angeles, has facilitated studies of histamine's role in gastric secretion. Of particular interest is their finding that gastrin has no effect on the parietal cell in the absence of histamine but becomes an extraordinarily potent (10^{-12}-10^{-8} M) stimulant of the parietal cell in the presence of trace concentrations (10^{-8}) of histamine. This finding places histamine's role in a different perspective than that proposed by earlier workers in the field. Studies in our laboratory with the elutriation technique have shown that histamine is located exclusively in cell fractions that have the characteristic morphology of mast cells. The histamine content of the purest fraction of these cells was 1.9 pg/cell. Preliminary studies have indicated that the amine is released by carbamocholine and prostaglandin E_1. The cells contain small amounts of the specific histidine decarboxylase. In contrast to histamine, serotonin is present in the larger of the two populations of chromaffin cells found in the dog gastric mucosa.

The concentration of histamine-N-methyltransferase in parietal cells is very high. Since a specific inhibitor of this enzyme would be useful in studies of histamine's role in gastric secretion, compounds similar in structure to histamine are being screened. Dimaprit, a recently introduced H_2 agonist, has been found to be an effective noncompetitive inhibitor ($K_m \sim 10^{-6}$ M) of

histamine-N-methyltransferase. Investigation of related compounds continues.

Studies of biogenic amines in disease: Collaborative clinical studies with the improved micromethods for the assay of biogenic amines and their metabolizing enzymes continue. In patients with various types of physically induced urticarias, histamine release into plasma was shown to be closely associated with the development and subsidence of symptoms in patients with cold-, cholinergic (exercise)- and vibratory-induced urticarias. Although histamine release was not detected in patients with other forms of physically induced urticarias, a different experimental approach suggests that histamine release may be involved in some of these disorders. Measurement of histamine in blister fluid (induced by mild vacuum applied to a plethysmograph) indicated abnormally high histamine levels in patients with bullous erythema multiforms, heat- and pressure-induced urticarias.

Studies establishing the utility of histamine measurements in plasma and urine by the enzymatic isotopic assay in the diagnosis of diseases associated with abnormal production are largely completed. Measurements have been made in samples obtained from over 200 patients with various disorders. These studies have identified the levels of histamine to be expected with the assay in urine and blood of control subjects, patients with mastocytosis and the various basophilic leukemias, and those with the various urticarial reactions discussed above. Abnormal histamine levels have been observed in no other conditions.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 02403-04 PB

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Isolation, Culture and Characterization of Lung Cells

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 MAN-YEARS:	PROFESSIONAL:	OTHER:
.7	.2	.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 objective of this study is to develop a technology to culture the cells of the lung that are important in the maintenance of normal parenchymal structure in health and disease. Using physical, enzymatic, and biological methods of isolation and culturing of lung cells, several cell strains have been developed including: fibroblasts from normal adult human lung, adult human lung from patients with a variety of lung disorders, fetal human lung, newborn rabbit lung, fetal rabbit lung, newborn rat lung, fetal cat lung, and adult rabbit skin. In addition, epithelial cells have been isolated from fetal cat lung and alveolar Type II cells have been isolated from the adult rabbit lung. Studies with some of these cells have demonstrated that lung fibroblasts maintain their differentiated state over many subcultivations. While fetal skin and lung fibroblasts are almost identical, they appear to differentiate with development so that they are quite different in the adult. Cell synchrony studies suggest that human fetal lung fibroblasts have an S phase of approximately 7 to 10 hours, A G1 phase of 10 to 12 hours and a G2 phase of approximately 3 hours. With these techniques, it should be possible to utilize sophisticated biochemical methodologies to evaluate the status of lung cells in human pulmonary disorders.

1123

Project Description:

Objectives: The use of biochemical technology to understand human lung disease is limited by the complexity of the organ, the unavailability of large quantities of lung cells, and the multitude of variables involved in attempting to study the lung in situ. Even though the biochemist can approach the study of lung cells with a vast armamentarium of concepts and sophisticated methodologies, the inherent complexity of dealing with multiple cell types immediately hinders the sorting out of the contribution of each cell type to the biochemical parameters being measured. A lung parenchyma devoid of blood elements and cells of the alveolus (a very difficult state to achieve experimentally) still has four major cell types (endothelial cell, mesenchymal cell, alveolar Type I cell, and alveolar Type II cell). In addition to the problems of cell purity, the study of lung cells in situ is compounded by the multitude of factors which impinge on each cell type to attempt to vary its inherent properties. Thus, to reduce the number of (mostly unknown) variables to a minimum, it has become necessary to develop techniques by which the individual populations of lung cells can be isolated, cultured and studied. One of the primary objectives of this laboratory is to develop a technology to culture the cells of the lung that are important in the maintenance of the normal parenchymal structure in health and disease. Of particular interest are those processes by which these cells synthesize and degrade the extracellular connective tissue matrix of the alveolus.

To this end, three approaches are being utilized: (1) establishment of cultures of different kinds of cells of human and animal lung; (2) evaluation of the differentiated state of cells in culture to be used for cell identification and for studying cell ontogeny; and (3) synchronization of cell cycles such that events in the life history of the cell can be studied in detail.

Methods: In general, there are three methods used for the isolation and culture of lung cells. The easiest is direct isolation, but this is limited to one cell type of the lung, the alveolar macrophage, the most available cell in the lung parenchyma. By bronchoalveolar lavage methods, 30 to 100 x 10^6 macrophages can be obtained per adult rabbit. Bronchoalveolar lavage in the normal human can yield 10 to 15 x 10^6 cells. These macrophages can be maintained in culture for 7 to 30 days. In culture, the macrophages adhere to surfaces, phagocytize, kill bacteria and release a wide variety of enzymes.

The second method used to isolate lung cells is by capitalization on specific cell physical and/or biological properties. Implicit in the definition of the "differentiated" cell is the concept that each cell population has different properties. Differences in density and/or adherence to surfaces are the most common parameters exploited in this type of cell isolation procedure. The methods used to group parenchymal cells in individual populations are just being refined. Before these cells can be grouped into individual populations, they must first be separated by enzymatic methods. Once free from the alveolar septum, the alveolar Type II cell can be substantially purified by allowing contaminating macrophages to phagocytize heavy materials which allow them to be removed from the Type II cells by sedimentation methods. The resulting Type II cells can then be analyzed and/or cultured.

The third method of isolation of specific lung cells are by culturing or by cloning methods. Basically, there are two variations of these methods. In the "hardiest will survive" method, the lung parenchyma is dispersed with enzymes and the resulting cells are allowed to grow in primary culture. Usually, one cell type will "overgrow" the plate. When confluency is reached, the cells are replated (passaged or subcultivated) and the hardiest cell population, usually a mesenchymal cell, obtains supremacy and eventually homogeneity. In the second variation of these methods, the cells are enzymatically dispersed from parenchyma and plated at very low cell densities. Populations of cells growing from one cell are recovered and allowed to grow to homogeneity.

Although the advantage of studying specific cell types in culture is apparent, there are major problems to be considered when using this technology to study the biochemical processes which maintain the lung structure and function. Using electron microscopic methods, it is relatively easy to identify the major cell types of lung when these cells are in situ. However, when these cells are in culture, many morphological features are lost; particularly the feature of anatomic location. To overcome the problem identification of lung cells in culture, we have utilized a method of displaying newly synthesized labeled secreted proteins on molecular weight "maps". These maps can then be compared from different cultures, cultures from different time and passage, cultures in different species, cultures in different organs, and cultures of varying age. This method can also be used to study the ontogeny of cells as they develop from fetal to adult life.

Four methods have been used to attempt to synchronize lung cells: (1) serum deprivation (stops cells in the early G1 state); (2) hydroxyurea treatment (stops cells at the beginning of S phase); (3) amethopterin treatment (beginning of S phase); and (4) double thymidine block (beginning of S phase). To follow the stages of cell cycle, several methods are used: (1) [^{3}H]-thymidine incorporation to study S phase; (2) audioradiography of labeled cells to determine the percentage of labeled mitoses; (3) cell number counts to detect doubling of cell population; and (4) light microscopic examination of Giemsa stained preparations to determine a mitotic index.

Major Findings: The culture techniques described above have been utilized to isolate and/or culture the following cell types: (1) alveolar macrophages from the adult human, adult rabbit and adult cat; (2) fibroblasts from normal adult human lung, adult human lung from patients with a variety of pulmonary disorders, fetal human lung, newborn rabbit lung, fetal rabbit lung, newborn rat lung, fetal cat lung, and adult rabbit skin; (3) epithelial cells from fetal cat lung; and (4) alveolar Type II cells from the adult rabbit lung.

These cells have been utilized in studies of the control of the synthesis and degradation of the extracellular matrix (See Project No. Z01 HL 02409-02 PB); studies of the hereditary disorders of connective tissue (See Project No. Z01 HL 02408-02 PB); and evaluation of the fibrotic lung diseases (See Project No. Z01 HL 02407-03 PB).

The method of molecular weight mapping of proteins secreted by cells in culture has been utilized to characterize different types of lung cells. The patterns of secreted proteins of the alveolar Type II cell, blood monocyte, alveolar macrophage and fibroblasts are completely different. These maps are characteristic of the cell types and can be used in addition to morphology to specify the types of cells in culture.

In addition, these molecular weight maps are being used to study the changes in the differentiated state of cultured cells. For example, we have used it to show that human fetal lung fibroblasts secrete the same proteins at subcultivation 3 as subcultivation 15, thus, demonstrating that the differentiated state is maintained during this period of time. However, even though the differentiated state for a given cell is maintained over many subcultivations, the source of the cell critically determines its differentiated state. In detailed studies of the ontogeny of the fibroblasts, we have demonstrated that the fetal lung and fetal skin fibroblasts are almost identical in terms of the proteins they secrete. However, the adult lung fibroblasts and adult skin fibroblasts, while still maintaining the differentiated characteristics of a "fibroblast", do show subtle but specific differences in their secreted proteins. Therefore, the development of the fibroblasts appears to be organ-specific, such that while identical in fetal life, fibroblasts differentiate as the animal matures.

Results of cell synchrony studies have shown that hydroxyurea and amethopterin are not effective in synchronizing human fetal lung cells. In comparison, serum deprivation apparently blocks these cells in early G1 phase. Upon addition of serum, the cells go through G1 and then to S but become somewhat asynchronous as they go through late G1. However, the method is reasonable for studying early to mid G1 phase. Double thymidine block is effective in synchronizing human lung cells at the G1-S interface. Following release of these cells they undergo a uniform wave of DNA synthesis of approximately 2 to 7 hours and then remain synchronous for about 12 hours. Thus far, the data suggests that for human fetal lung fibroblasts (doubling time approximately 25 hours), the S phase is approximately 7 to 10 hours. G2 is unclear but possibly about 3 hours. Mitosis unknown but should be one hour or less and G1 is about 10 to 12 hours.

Significance to Biochemical Research and Institute Program: The mechanisms by which the differentiated state is controlled in the lung are the mechanisms by which the lung maintains its structure and function. In the fibrotic lung disorders, for example, these mechanisms are presumably deranged so that connective tissue is synthesized in an abnormal fashion. Establishment of diploid cell lines of the cells responsible for the maintenance of the alveolar structure in health and disease allows investigation at the molecular level. These types of studies include identification of the normal mechanisms of lung and how these mechanisms are varied following lung injury. It is now possible to culture cells from a variety of human lung disorders which should allow the utilization of sophisticated biochemical methodologies to studying the status of pulmonary cells in these diseases.

Proposed Course to Project: There will be continued establishment of different cell lines, the investigation of various proteins and other macromolecules made by these cells and evaluation of the mechanisms of the maintenance of the differentiated state. One area of particular interest is the control of synthesis and degradation of connective tissue by the cells. These mechanisms involve either the inherent properties of these lung cells or by cell-cell interactions between different forms of cells of the lung as well as blood derived cells that are brought to the lung. As these techniques are established in normal animal lung cells they are applied to the study of human lung cells from both normals and patients with a variety of lung disorders.

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Hance, A.J., Horwitz, A.L., Cowan, M.J., Elson, N.A., Collins, J.F., Bienkowski, R.S., Bradley, K.H., McConnell-Breul, S., Wagner, W.M., and Crystal, R.G. Biochemical Approaches to the Investigation of Fibrotic Lung Disease. Chest, 69 (Suppl.), 257-261, 1976.

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Elson, N.A. and Crystal, R.G. Approaches to the Study of Environmental Pollutants. In: S.D. Lee (ed.). Biological Effects of Environmental Pollutants, Ann Arbor Science Publishers, 1977 (in press).

PROJECT NUMBER (Do NOT use this space)	HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	Z01 HL 02405-04 PB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Experimental Models of Pulmonary Fibrosis

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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Pulmonary Branch

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

To evaluate the importance of the immune system in the pathogenesis of pulmonary fibrosis, the susceptibility of the nude, athymic mouse to fibrosis was tested by the administration of bleomycin, a drug which produces a high incidence of interstitial pneumonitis and fibrosis. Bleomycin administration resulted in pulmonary lesions of widely varying severity in both normal and nude mice. Both developed pulmonary fibrosis and pneumonitis; there were no differences in the susceptibility of the control mice and nude mice to bleomycin. To the extent that the athymic mice and the amounts of bleomycin used in this study are valid representations of pulmonary fibrosis in man, the importance of the cellular immune system in the pathogenesis of the fibrotic lesion has to remain questionable.

1128

Project Description:

Objectives: The classification of the fibrotic lung disorders contains approximately 130 diseases, including many of known etiology as well as those with distinct characteristics but unknown cause. As diverse as these disorders may be, they have common features which suggest that pulmonary fibrosis follows a pattern of parenchymal injury, inflammation, and cellular and extracellular matrix disordering. In most of these disorders, the factors which influence the outcome of this general pattern include the nature of the primary agent, the severity of the injury, and various genetic and acquired host factors.

A growing body of evidence suggests that the immune system is one host factor which may influence the development of pulmonary fibrosis. However, the description of unusual immune processes in these patients only yields indirect evidence as to their importance; it does not indicate whether the immune system participates in the development of the fibrotic lesion.

To evaluate the importance of the immune system in the pathogenesis of pulmonary fibrosis, we have asked whether selective deletion of parts of the immune system change host susceptibility to pulmonary fibrosis. We have begun by examining T-cell dependent cellular immunity, and have chosen the nude, athymic mouse as a model of selective deficiency of this component of the immune system. The susceptibility of this mouse to fibrosis was tested by administration of bleomycin, a drug which produces a high incidence of interstitial pneumonitis and fibrosis. Bleomycin is particularly suited for a study evaluating the importance of immune mechanisms in the development of pulmonary fibrosis since it causes little, if any, immunosuppression.

Methods: Two groups of mice were evaluated. Normal mice from an outbred colony of Swiss-type mice established at NIH (N:NIH[S]). Nude, athymic mice were from an outbred colony with the same genetic composition as the N:NIH(S) mice except for the presence of the homozygous nude mutation.

Bleomycin sulfate was given in various dose schedules previously established to give pulmonary fibrosis and pneumonitis in a large percentage of the control mice. Two to four weeks after receiving the course of bleomycin, the mice were sacrificed, the lungs removed and fixed in inflation with neutral formalin. These fixed, inflated lungs were then evaluated using light microscopic methods. The presence of fibrosis and pneumonitis were graded at a scale from 0 to +3 using criteria previously established in this laboratory.

Major Findings: Bleomycin administration resulted in pulmonary lesions of widely varying severity in both the normal and nude mice. Both exhibited focal, pleural-based lesions consisting of pleural thickening, disorganization of the alveolar architecture, mononuclear interstitial infiltration and accumulation of interstitial collagen. There were no differences in the susceptibility of the control mice and nude mice to bleomycin.

Significance to Biochemical Research and Institute Program: The hypothesis that the immune system is an important host factor modulating the development

of pulmonary fibrosis is based on circumstantial evidence associating abnormal immune phenomena with the presence of the fibrotic lesion. While this clinical information implicates the immune system in the pathogenesis of the pulmonary fibrosis, it does not indicate the importance of immune processes in the development of this disease. These immune phenomena, however suggestive, may only be bystander reactions, elicited by the presence of altered tissue components at the site of parenchymal lung injury.

The use of the T-cell depleted mouse as a model of the development of pulmonary fibrosis is an attempt to directly evaluate the importance of the immune system in the evolution of the fibrotic lesion. Since the mouse lacking functioning T-cells develops pulmonary fibrosis in response to bleomycin as readily as the normal mouse, these data suggest an intact cellular immune system is not a necessary condition for the development of pulmonary fibrosis.

However, these findings do not completely rule out the possibility that cellular immune pnenomena are important in the development of pulmonary fibrosis. It is important to recognize that the lesion of bleomycin induced pulmonary fibrosis in the mouse may be different from that in man; particularly since the dose levels used in the study were 50 times that considered to be "fibrogenic" in man. In addition, bleomycin may not be a representative model for most of the other approximately 130 forms of fibrotic lung disease. It is also possible that the fibrosis associated with bleomycin may normally be mediated through cellular immune processes but in their absence alternative pathways can cause the same lesion. It is also recognized that while the nude athymic mouse is an excellent model of T-cell depletion, it does not represent an absolute deletion of all cell-mediated processes; it is conceivable that the specific cell-mediated processes critical to bleomycin to induce fibrosis are still functioning in this mouse.

However, to the extent that the athymic mouse and the amounts of bleomycin used in this study are valid representations of pulmonary fibrosis in man, the importance of the cellular immune system in the pathogenesis of the fibrotic lesion has to remain questionable.

Proposed Course to Project: The use of a specific fibrogenic agent and carefully defined animal models with specific immune differences are powerful tools for understanding the pathogenesis of pulmonary fibrosis. Work is ongoing to evaluate the possibility that the H-2 locus (the histocompatibility locus in the mouse) may be associated with the susceptibility to the development of pulmonary fibrosis. To this end, a variety of mice with different genetic backgrounds and different H-2 types (a,b,d,k,q) are being evaluated for susceptibility to pulmonary fibrosis.

If mice can be identified that are more or less susceptible than other mice to the development of this lesion, this will provide an excellent investigative tool to understanding those processes which contribute to the disease in man.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02407-03 PB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Clinical Studies of Fibrotic Lung Disease

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	2.3	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)

Studies in patients with idiopathic pulmonary fibrosis have demonstrated: (1) the disease process not only involves parenchyma but also small airways as demonstrated by correlative physiologic and morphologic studies; (2) the best predictor of degree of fibrosis is the drop in the arterial PO_2 per unit work done; (3) the majority of patients with idiopathic pulmonary fibrosis develop pulmonary hypertension mid-course in their disease; (4) the degree of parenchymal information can be predicted with the ^{67}Gallium scan; (5) ventilation-profusion studies have demonstrated marked abnormalities in ventilation-profusion ratios; together with mixed venous blood gases this data can be used to predict arterial blood gases within 5%; (6) lavage of the lower respiratory tract demonstrated elevated levels of IgG but normal ratios of T and B cells; (7) lavage fluid analysis have also shown these patients have elevated levels of collagenase, neutral protease and β-glucuronidase but not elastase; (8) they have normal phenotype frequencies of HLA-A and B antigens; (9) peripheral lymphocytes from these patients recognize collagen as "non-self"; and (10) an ongoing double-blind study of treatment of this disease with steriods and asothioprine now has 30 patients and should be completed within the next year.

1131

Cooperating Units:

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Project Description:

Objectives: The fibrotic lung disorders represent 15 to 20% of the non-infectious disorders of the lung. There are more than 130 separable disease entities associated with pulmonary fibrosis, but they can be categorized into ten general groups (occupational and environmental etiologies, drug reactions, physical agents, infectious disorders, secondary to cardiac disease, pulmonary vascular disease, neoplastic disease, congenital and familial, unknown etiology but with characteristic morphology, idiopathic pulmonary fibrosis). The Pulmonary Branch, NHLBI, has undertaken a detailed study of patients with pulmonary fibrosis, particularly those with environmental etiologies and those with idiopathic pulmonary fibrosis (IPF). The latter represents a chronic, devastating illness, invariably resulting in death an average of 4 to 5 years from the onset of symptoms. Although approximately 5 to 10% of patients with IPF respond to corticosteroids, there is no known treatment for the remainder. The objectives of our studies are as follows: (1) to determine the etiology of IPF; (2) to follow patients with IPF longitudinally to determine the natural history of the disorder and which pulmonary function parameters are most sensitive to the disease process; (3) to correlate pulmonary alveolar constituents (alveolar lavage fluid and cells) in IPF with histopathology and pulmonary function parameters as well as with alveolar constituents in other fibrotic lung disorders; (4) to correlate radioisotopic monitors of lung function (ventilation, perfusion and gallium scans) with the disease process; (5) to correlate the histopathology in IPF with biochemical and functional parameters; (6) to study the therapy of IPF; (7) to study abnormal inflammatory and immunologic processes associated with the continued pathogenesis of the IPF; and (8) to study genetic determinants of IPF.

Methods: Patients admitted to the Pulmonary Branch Clinical Service enter an extensive protocol which includes: detailed medical and pulmonary history and physical exam, routine serologic, roentgenographic and EKG studies, serologic studies aimed at immune processes; pulmonary function studies including lung volumes, flow rates, diffusing capacity, flow-volume curves, closing volume, closing capacity, body plethysmograph functional residual volume and airway resistance, static and dynamic pressure-volume curves, ventilatory and arterial blood gas studies at rest and exercise, lung lavage for cellular function and morphology, secreted proteins of macrophages and other constituents of alveolar wash fluid, technetium vascular scans, ^{133}Xe ventilation scans and $^{85}Gallium$-citrate scans. In some patients the pulmonary circulation is evaluated with right heart catheterization. When indicated, lung biopsy is done either through the fiberoptic bronchoscope or via open thoracotomy. Tissue is studied by light microscopy, electron microscopy, culture (routine, fungal, mycobacteria and virus), collagen synthesis and degradation, and evaluation of the cellular components of the inflammatory and immune systems. Peripheral blood lymphocytes are isolated with dextran or Ficoll-Hypaque methods and are tested in vitro for interrelationships to constituents of the alveolar interstitum including purified components of the extracellular matrix as well as cells obtained at open lung biopsy. Selected patients are entered into a drug treatment protocol where all patients are treated with corticosteroids (standard therapy) and 50%

of the patients are treated (in addition) with azathioprine in a double-blind fashion.

Major Findings: Studies in patients with idiopathic pulmonary fibrosis have demonstrated the following:

(1) A significant number of patients with this disease demonstrate abnormalities in frequency dependency of compliance, flow-volume curves, and maximum flow-static recoil curves. These abnormalities correlate with the morphological assessment of small airways of patients with this disease. This data demonstrates that part of the pathogenesis of idiopathic pulmonary fibrosis is in the small airways, as well as in the pulmonary parenchyma. It gives a rational for part of the hypoxemia of the disease as being related to ventilation-perfusion abnormalities secondary to airflow problems, rather than derangement of the alveolus alone.

(2) It has been known for some time that patients with idiopathic pulmonary fibrosis reduce their arterial PO_2 with exercise. Correlative studies of the morphologic assessment of fibrosis in lung biopsies from these patients with quantitative assessment of the drop in the arterial PO_2 per unit of work done, demonstrates a striking correlation. In comparison, the conventional monitors of pulmonary function for these patients (total lung capacity, vital capacity, diffusing capacity) do not correlate with the degree of fibrosis in the open lung biopsies at all. In addition, detailed analysis of the static deflation volume-pressure curves of patients with idiopathic pulmonary fibrosis show that several derived parameters from this curve also correlate with the degree of fibrosis on open lung biopsy. With these tools, the clinician now has a means to assess the amount of fibrosis that these patients have.

(3) A significant number of patients with idiopathic pulmonary fibrosis in mid-course develop pulmonary hypertension. Studies of the pulmonary circulation of these patients compared to a variety of pulmonary function parameters and histological evaluation of the pulmonary arteries have demonstrated: (a) all patients with diffusing capacity of less than 45% predicted have pulmonary hypertension; (b) total lung capacity and vital capacity have no correlation with the degree of pulmonary hypertension; (c) in general, as the arterial PO_2 falls, the pulmonary artery pressure rises, but it is not a direct correlation; (d) the degree of pulmonary hypertension in these patients can be predicted by emperic equations relating pulmonary function parameters; (e) there is a correlation between the number of pulmonary vessels narrowed in the open lung biopsy and general level of pulmonary hypertension; and (f) in addition to pulmonary hypertension, some patients with idiopathic pulmonary fibrosis seem to have right ventricular dysfunction as evidenced by a decreased cardiac output and elevated right ventricular end-diastolic pressure when the mean pulmonary pressure is greater than 35.

(4) ^{67}Gallium citrate scanning in patients with idiopathic pulmonary fibrosis have demonstrated that 70% of these patients will accumulate this isotope in their lung parenchyma four to eight hours after injection. A method has been

developed (termed the ^{67}Gallium index) by which the uptake of gallium in these patients can be quantitated in terms of the area involved, the intensity of the uptake, and the texture of uptake. Correlation of this numerical data with the degree of cellularity on open lung biopsy and the analysis of cells recovered by bronchoalveolar lavage demonstrated that the gallium scan is an accurate predictor of the degree of the parenchymal information in these patients.

(5) Scintagraphic studies with macroaggregated albumin and ^{127}Xenon have demonstrated that patients with idiopathic pulmonary fibrosis have marked abnormalities in both ventilation and perfusion. These studies have been correlated using mathematical and computer methods to produce ventilation-perfusion ratios for each of 1,500 to 2,000 small regions of lung. It is now possible to evaluate which areas of lung are functioning normally and which areas are contributing to the patient's hypoxemia. In addition, these studies have been correlated with data obtained at right heart catheterization to demonstrate that with central venous blood gases and the ventilation-perfusion scan we can now accurately predict the arterial blood gas levels within $\pm$ 5%. Thus, it is now possible to determine the amount of oxygen being transferred in each of many small regions of lung.

(6) The fiberoptic bronchoscope has proven to be a valuable tool in the analysis of the cellular constituents of the lower respiratory tract. Studies of patients with idiopathic pulmonary fibrosis have demonstrated that they have elevated levels of neutrophils in their lower respiratory tract as well as elevated levels of IgG. Preliminary studies on the types of lymphocytes found in the lower respiratory tract have demonstrated that they have a normal ratio of T-cells and B-cells. This is in comparison to patients with chronic hypersensitivity pneumonitis (farmer's lung) that have a markedly elevated ratio of T-cells to B-cells in their lungs. Prospective studies of bronchoalveolar lavage in patients with idiopathic pulmonary fibrosis suggest that this technique may be useful for determining efficacy of anti-inflammatory therapy on the disease process.

(7) The fiberoptic bronchoscope has also been utilized to recover the fluid bathing the epithelial surface of the lower respiratory tract of patients with idiopathic pulmonary fibrosis. Analysis of enzyme constituents of this fluid have shown that while these patients have normal levels of LDH and lysozyme, they have significantly elevated levels of neutral protease and collagenase. They do not, however, have any elastase. In addition, they have significantly elevated levels of ß-glucuoroidase. Since we know that the alveolar interstitium in these patients is "disordered", these findings give a rational basis for the morphological disordering.

(8) Studies of the phenotype frequencies of the A and B locus antigens of the HLA system in patients with idiopathic pulmonary fibrosis have demonstrated there is no definite statistical difference in the distribution of these antigens between idiopathic pulmonary fibrosis and healthy controls. Thus, although there are many clues that this is a disease with a genetic basis, this data suggests that the genetic determinants controlling susceptibility and/or

course of the disease are either far removed from the HLA system or that the disease is one that is polygenetic.

(9) In vitro evaluation of the peripheral lymphocytes of patients with idiopathic pulmonary fibrosis has demonstrated that these cells view collagen as "non-self". Since it is the collagen that is disordered in the alveolar interstitium in this disease, this suggests that cell-mediated and immune mechanisms may play a role in the continued pathogenesis of the disease.

(10) Antibodies against specific collagen types have been developed in order to evaluate serum and alveolar lavage fluid or the presence or absence of anti-collagen antibodies.

(11) Approximately 50% of patients with idiopathic pulmonary fibrosis have elevated levels of cryoglobulins. It is not clear what these cryoglobulins are composed of, but the two possibilities exist: (a) they may represent immune complexes; or (b) they may represent elevated levels of cell surface protein in the form of cold insoluble globulin (See Project No. Z01 HL 02411-01 PB). If they represent immune complexes, this would suggest that the humoral immune system may be involved in the pathogenesis of the disease. If the cryoglobulin represent cold insoluble globulin, this may be a marker for the relative "disordering" of lung cells in this disease.

(12) To test the validity of the suggestion that cell-mediated immune phenomena are involved in the pathogenesis of idiopathic pulmonary fibrosis, patients with this disease are being skin tested with purified preparations of animal and human collagens to determine whether they manifest hyperreactivity to these antigens as mediated through the cellular immune system.

(13) This represents the second year of the double-blind study of the treatment of idiopathic pulmonary fibrosis with azathioprine (all patients are on corticosteroids with 50% on azathioprine in a blind fashion). To date, 30 patients have been enrolled in this study and it is projected that within the next year the data will approach significance such that the blind can be broken and it can be firmly established as to whether there are differences in this drug therapy for this disease.

(14) Evaluation of open lung biopsy specimens of patients with idiopathic pulmonary fibrosis have revealed that they have normal concentrations of collagen and normal rates of collagen synthesis. Although surprising at first, this data suggests: (a) the disease is one of rearrangement rather than just laying down a more fibrous tissue; and (b) drugs that inhibit the synthesis of collagen may not be useful in treating these patients.

(15) A large body of clinical radiographic, physiologic, morphologic, and scintagraphic and biochemical data is accumulating on these patients. They are being analyzed by mathematical models to develop emperic equations of predictors of the pneumonitis and fibrosis of this disease. With these equations plus conventional monitors of disease process, it may be possible

to predict what a biopsy would show without resorting to open lung biopsy. In addition, analysis of this data will provide assessment of predictors of the clinical course of these patients which will aid physicians in evaluating and advising the patients and families.

Significance to Biomedical Research and Institute Program: The fibrotic lung disorders are almost uniformly fatal and affect a significant proportion of the population. Up to this time, there has been no information on pathogenesis and there is essentially no cure. By combining studies of patients with these disorders with our large basic research program concerning the control of connective tissue composition and accumulation in lung, we expect to make major inroads into understanding and treating these disorders.

Proposed Course to Project: 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 lung collagen synthesis and degradation can be studied with pharmacologic agents. Immunologic studies will continue to explore cell-mediated immune mechanisms in these disorders. As results with pharmacologic agents become promising, they will be studied in patients when applicable.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02408-02 PB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Clinical Studies of Hereditary Lung Disease

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

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(See Attachment)

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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)

Several forms of hereditary lung disease are being used to help understand the structure and function of normal lung. Patients with hereditary disorders of connective tissue are being evaluated by pulmonary function testing to make inferences of the mechanical-biochemical correlates of lung structure and function. The homozygous form of α1-antitrypsin deficiency associated with emphysema is being used to evaluate the role of antiproteases in protection of lung disease. Studies with a new drug, Danazol, has shown that α1-antitrypsin levels in these patients can be increased by 50%. Familial fibrotic lung disease is being evaluated as a model to understand the genetics of idiopathic pulmonary fibrosis. The homozygous forms of sickle cell disease is being used as a model to evalute the effect of manipulation of the oxygen hemoglobin-dissociation curve on exercise tolerance. Fabry's disease is being evaluated as a model for the effect of inherent abnormalities of airway cells on the development of obstructive lung disease.

1139

Cooperating Units:

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D. Miller, Clinical Associate, Hematology Branch, NHLBI

Project Description:

Objectives: There are several forms of lung disease which have a known hereditary basis. By evaluating these "experiments of nature" we can derive an understanding of the structure and function of lung in health and disease which can then be applied to a general understanding of pulmonary disorders. The following disorders have been chosen as models of more general forms of lung disease: (1) hereditary disorders of connective tissue (Marfan's Syndrome, Ehlers-Danlos Syndrome, and Osteogenesis Imperfecta) are evaluated to develop an understanding of the contribution of different components of connective tissue to the structure and function of lung; (2) the homozygous form of α1-antitrypsin deficiency associated with emphysema is used to evaluate the general concepts of destructive lung disease; (3) familial fibrotic lung disease is indistinguishable from idiopathic pulmonary fibrosis; an understanding of the genetics of this disease is used as a model for the genetics of idiopathic pulmonary fibrosis; (4) the homozygous form of sickle cell disease is used as a model to evaluate the effect of manipulation of the oxygen-hemoglobin dissociation curve on exercise tolerance; and (5) Fabry's Disease (Angiokeratoma Corporis Diffusion Universlae) is used as a model for evaluating the effect of abnormalities in airway cells in the development of obstructive lung disease. The objectives of each of these studies are as follows:

(1) Hereditary disorders of connective tissue. The connective tissue of lung is complex, consisting of four genetically distinct types of collagen, elastic fibers (elastin together with microfibrils), seven types of glycosaminoglycans with their associated proteins and a variety of other macromolecules composing the ground substance. This complexity, together with the intricate anatomy of lung parenchyma, makes the study of the influence of connective tissue on the control of lung structure and function extraordinarily difficult. The major objectives of this proposal are attempts to use "experiments of nature" involving connective tissue to help understand the role each connective tissue component plays in the maintenance of lung structure and function. The lung is the only organ of the body where it is relatively easy (using the sophisticated methodology of pulmonary function testing) to make direct inferences on the role of connective tissue in controlling mechanical properties. Thus, the lung affords an opportunity for understanding the biochemical-mechanical correlates of connective tissue. This project is done in conjunction with biochemical studies on the control of synthesis and destruction of connective tissue (See Project No. Z01 HL 02409-02 PB). Patients with specific connective tissue disorders are admitted to the Pulmonary Branch Clinical Service and sophisticated methods of pulmonary function testing are used to evaluate the "mechanical" status of their lungs. This is a long-term project but with the methodology available today, it should be possible to specifically define the role of each connective tissue component in lung and demonstrate how these components describe pulmonary abnormalities in these hereditary disorders.

(2) α1-antitrypsin deficiency is a genetic disease in which those affected persons have a striking reduction of this serum glycoprotein by both functional and antigenic criteria. Normally, α1-antitrypsin is one of the principal

components of the serum protease inhibitor system. In the homozygous difficiency state (phenotype "ZZ"), 80 to 90% of affected adults have accelerated, usually fatal, obstructive pulmonary disease. Although considerable progress has been made in characterizing this protease inhibitor abnormality, clues to the precise pathophysiology and effective therapy for this disorder are lacking. Current indications suggest the ZZ homozygous protein is a single amino acid substitution. Most likely, this abnormality results in changes in intracellular confirmation of the molecule such that sugar sidechains cannot be added. Consequently, the altered ZZ protein cannot be secreted in a normal fashion by the liver cells in which it is made. Recently, the Laboratory of Clinical Investigation, NIAID, has used the drug Danazol [17α-pregnene-20-yno-(2,3-d) ixoxazol-17-01] in the therapy of hereditary angioneurotic adema. Not only has this agent proved useful in the management of this clinical syndrome, it also seems to have reversed the associated biochemical defect (decreased levels of the C1 esterase inhibitor). Since the C1 esterase inhibitor is also an antiprotease made by the liver, the biochemical basis of hereditary angioneurotic adema may be very similar to that of α1-antitrypsin deficiency. For this reason, a drug trial with Danazol was started in the ZZ homozygous patients.

(3) Several families have been identified in which the clinical radiographic, physiologic, and morphologic findings are identical to idiopathic pulmonary fibrosis. Although the genetics of this disease is unclear, it is an autosomal defect and not sex-linked. The purpose of evaluating these patients is to understand the genetics of the disease in a fashion such that markers may be developed that could then be applied to the evaluation of genetic susceptibility to the development of idiopathic pulmonary fibrosis.

(4) It has been known for some time that shifting the oxygen-hemoglobin dissociation curve of patients homozygous for sickle cell disease to the left reduces the ability of the red cells to sickle. The Hemotology Branch, NHLBI, has an ongoing study to determine whether shifting the oxygen-hemoglobin dissociation curve of these patients in vivo might reduce the number of sickle cell crises. This is being accomplished by a variety of means, including bicarbonate therapy. Although it is possible that such therapy might reduce the number of sickle cell crises, it is also possible that manipulation of the oxygen-hemoglobin dissociation curve may change the ability for oxygen to be transferred to peripheral cells in a normal fashion. In a collaborative study, the Pulmonary Branch and Clinical Hemotology Branch of NHLBI are evaluating these patients before and after bicarbonate therapy to determine whether or not altering the position of the oxygen-hemoglobin dissociation curve will alter the ability of these patients to exercise.

(5) The sphingolipid disorders are lipid storage diseases that result secondary to a deficiency in an enzyme necessary for sphingolipid metabolism. The characteristic organ deposition of the lipid determines the clinical course of the specific storage disease. Fabry's Disease, Angiokeratoma Corporis Diffusion Universlae, is a sex-linked sphingolipid disorder. The lipid which accumulates is ceramide trihexoside and the deficient enzyme is ceramide trihoxosidase. The deposition of the lipid characteristically results in skin lesions,

peripheral neuropathy, ocular lesions, cardiovascular dysfunction and renal failure. Pulmonary involvement has generally been considered to be a component in this lipid storage disorder. When preliminary studies in our laboratory demonstrated that these patients had characteristic lipid disorders in the epithelial cells lining the airway, this study was developed in conjunction with MNB, NINCDS, to evaluate the extent of the airway disease in these patients. Specifically, we plan: (a) to determine the extent of airway disease in these patients and (b) correlate these functional abnormalities with morphological and biochemical evaluation of the airway cells themselves.

Methods: The clinical methods used to evaluate all of these patients are similar to that described in Project No. Z01 HL 02407-03 PB for the fibrotic lung disease protocol. In addition, the following specific methods were used for each of these projects:

(1) Patients admitted to the Clinical Service, Pulmonary Branch, who have hereditary disorders of connective tissue are fully evaluated using a number of pulmonary function methods. This can be a problem with a number of these patients, particularly those with osteogenesis imperfecta, because of the fragility of their bones and the fact that many of them cannot exercise. The functional abnormalities determined in these studies are compared with similar studies using patients with other forms of lung disease as well as healthy controls.

(2) Hospitalized patients with α1-antitrypsin deficiency (Type ZZ) are given 200 mg Danazol p.o., t.i.d. for 30 days. Serum protease inhibitor levels (α1-antitrypsin, α2-macroglobulin and C1-esterase inhibitor) are quantitated on alternate days for the duration of the 30-day treatment.

(3) Patients with familial fibrotic lung disease are typed for the major histocompatibility locus as well as the D-locus using conventional methodologies.

(4) Patients with sickle cell disease are exercised using graded treadmill exercise before and after bicarbonate therapy. The extent of exercise, as well as several derived parameters, are correlated with the changes in the oxygen-hemoglobulin dissociation curve.

(5) Patients with Fabry's Disease undergo usual pulmonary function tests as well as general clinical evaluation. They are then bronchoscoped with the fiberoptic bronchoscope and bronchial cells are obtained by lavage and brushing. The cells are evaluated by morphological methods using light and electron microscopy. In addition, quantitative lipid determinations are done by thin layer chromatography.

Major Findings: A number of aspects of this work are just beginning and the past year has been spent primarily in developing the methodologies to define the studies. The following specific information has been gained:

(1) Several patients have been evaluated with osteogenesis imperfecta. These patients have severe chest deformities secondary to multiple rib fractures,

making their evaluation very difficult. We are continuing to accumulate data from these patients as well as from individuals with Marfan's Syndrome. As patients become available with the various forms of Ehlers-Danlos Syndrome, they will also be evaluated.

(2) The studies with Danazol therapy of the ZZ homozygous group of α1-antitrypsin deficiency have shown that Danazol will increase the levels of α1-antitrypsin in the serum of these patients by 50% to 80% of the patients. Whether or not the increases from an average of 30 mg % to 45 mg % will be efficacious for the therapy of this disease will require a great deal of further detailed study.

(3) The genetic studies in the familial fibrotic lung disease are still ongoing.

(4) Several patients with sickle cell disease that have received therapy to shift their oxygen-hemoglobin dissociation curve have been evaluated before and after bicarbonate therapy by detailed exercise testing. Results are still being analyzed.

(5) Preliminary studies of Fabry's Disease clearly show that there is abnormal lipid deposition in the bronchial cells lining the large airways. The study is now in the process of accumulating data so that quantitative analysis may be done.

Significance to Biochemical Research and Institute Program: Several of the studies outlined are long-term studies but are of fundamental significance in the understanding of the structure and function of lung in health and disease. The connective tissue of lung is so complex that it would be difficult to determine the role of each component of the interstitial connective tissue unless methods such as those described above can be utilized. As more patients are evaluated, patterns should emerge which help to determine the contributions of each component of connective tissue as they relate to pulmonary structure and function.

The studies of α1-antitrypsin therapy with Danazol is the first example of the "successful" treatment of this disease. Whether or not the levels of α1-antitrypsin achieved with this drug will significantly effect the outcome of this disorder will take detailed studies. However, the finding is fundamental in that it clearly states that it is possible to raise the levels of α1-antitrypsin in this disorder with long-term therapy.

An understanding of the genetics of familial lung disease should enable us to make inroads into understanding why some people are susceptible to idiopathic pulmonary fibrosis and why others do not get the disorder.

One of the serious possibilities of therapy for sickle cell disease is manipulation of the oxygen-hemoglobulin dissociation curve such that patients are less susceptible to sickling phenomena. However, this manipulation may be detrimental to patients (or better) in terms of their oxygen delivery systems. This study will help understand the implications of shifting the

oxygen-hemoglobin dissociation curve in this disease and others.

Not only will analysis of the lipid deposition in Fabry's Disease in bronchial cells add a new dimension to this disorder but the current assay for enzyme levels in this disease are done by kidney biopsies. Bronchial brushing procedures are much more benign than renal biopsy and thus this methodology may prove useful in following these patients.

Proposed Course to Project: These clinical studies will continue as outlined above. The studies in Fabray's Disease, hereditary disorders of connective tissue, familial fibrotic lung disease and sickle cell disease are long-term projects. The α1-antitrypsin therapy project will be expanded to include study of heterozygotes. This is of particular importance because it has been suggested that patients heterozygous for the Z gene may be more at risk for the development of emphysema. Since Danazol is a relatively inoculous drug, it may bring the serum level of α1-antitrypsin above the threshold level by which these patients are more at risk. We are also doing preliminary studies to determine whether or not patients with α1-antitrypsin may be treated by the intravenous replacement of the deficient protein. There is information available suggesting that the half-life of the normal protein is approximately 5 days. It may, therefore, be possible to treat patients with α1-antitrypsin deficiency by intravenous replacement in the same manner that hemophilia is treated.

Publications: None

PROJECT NUMBER (Do NOT use this space)

HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

Z01 HL 02409-02 PB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Control of the Synthesis and Degradation of the Extracellular Matrix

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8.0	4.2	3.8

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)

These studies are designed to evaluate control of the synthesis and degradation of the extracellular matrix particularly as related to collagen. Studies with cultured lung cells demonstrated that both epithelial and mesenchymal cells synthesize collagens Types I and III. These cells do so at a constant rate over extended periods of time. Explants of human lung from patients with idiopathic pulmonary fibrosis disease have shown that these tissues synthesize the same amounts of collagen per cell as do normal tissue, suggesting this disease is one of derangement rather than that of excess collagen. Evaluation of immune and hormonal controls of collagen synthesis suggests that it is possible to regulate the differentiated state of collagen synthesis by external manipulations. Evaluation of collagenases by lung cells have shown that the neutrophil produces a collagenase specific for Type I cells. In addition, cells such as macrophages produce neutral proteases which activate latent collagenases secreted by the same cell. Studies of lung explants have demonstrated that 30 to 40% of newly synthesized collagen is degraded intracellularly. This appears to be independent of other known mechanisms of collagen degradation. Type specific antibodies against collagen Types I and III show that human fetal fibroblasts synthesize both.

Continuation of Other Professional Personnel Engaged on the Project:

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Project Description:

Objectives: The structure and function of lung is critically dependent on the organization of the extracellular matrix. This matrix is composed of collagen, elastic fibers, proteoglycans, and the remainder of the ground substance, including a number of large macromolecules, degradative projects of cells and connective tissue, and serum constituents. The maintenance of this extracellular matrix is the responsibility of the lung cells within and bordering the interstitium. The types, quantities, and locations of the connective tissue constituents that these cells synthesize and degrade is dependent on the inherent differentiated properties of these cells as modulated by cell-cell interactions as well as hormonal, immune and inflammatory processes.

The purposes of these studies are to evaluate the control of the synthesis and degradation of the extracellular matrix of lung. Although specifically directed toward lung, the implications from a number of these studies are broad-based and apply to many organs of the body.

Our studies in the past year have been directed primarily at collagen, the major component of this extracellular matrix. Specifically, these studies have been designed to evaluate: (1) the types of collagen synthesized by each cell type in the lung; (2) how well lung cells maintain their differentiated state in regards to the synthesis of collagen; (3) quantitation of the rates of synthesis of collagen by explants of lung from normal humans compared to patients with fibrotic lung disease; (4) immunologic control of collagen synthesis; (5) hormonal control of collagen synthesis; (6) post-translational modifications of collagen; (7) quantitation of collagen messenger RNA and detailed studies of mechanisms of control of collagen synthesis at the gene level; (8) the destruction of collagen by collagenase; (9) the intracellular destruction of collagen; and (10) the use of antibodies directed against specific collagen types to evaluate cell culture, human biopsy material, circulating antibodies in patients and humoral immune mechanisms which may change the differentiated state of lung cells.

Methods: The majority of the methods used for these studies have been developed in this laboratory over a number of years. Many utilize lung cells in culture (See Project No. Z01 HL 02403-04 PB). The specific methods used for each of these studies are as follows:

(1) To evaluate the collagen type synthesized by specific cells, cultures are incubated with labeled amino acids and the media collected after a period of time. The newly synthesized collagens in the media are then purified by a variety of chromatographic techniques and the identity of the collagens evaluated using cyanogen bromide cleavage methods followed by column chromatography and polyacrylamide gel electropholesis.

(2) Two approaches have been utilized to evaluate the maintenance of the differentiated state in tissue culture in regards to collagen synthesis. First, HFL-1, a human fetal lung fibroblast extensively characterized in this

laboratory, have been evaluated at subcultivations 5 through 25 for the rates of collagen synthesis, rates of non-collagen protein synthesis and percent collagen synthesis. Second, cultures of NB-6, a newborn rabbit lung fibroblast, have been evaluated for the relative amounts of Type I and Type III collagen in that they synthesize. The latter is done using cyanogen bromide peptide mapping techniques.

(3) The rates of synthesis of collagen in human lung explants is carried out in short term cultures using amino acids to label the collagen. By quantitating labeled collagen and DNA in the cultures an estimate can be made of the average rate of synthesis of collagen.

(4) To determine whether or not cell-mediated immune mechanisms may have an influence on the differentiated state of fibroblasts in regards to collagen synthesis, lymphocytes from normal humans are stimulated with phytohemagglutinin for several days and the supernatants removed and added to cultures of human fetal lung fibroblasts for two days. Using appropriate nonstimulated controls, the fibroblasts are then evaluated for their ability to synthesize collagen.

(5) To evaluate the possible hormonal modulation of collagen synthesis, fibroblasts are incubated with various hormones known to raise intracellular cyclic AMP. Cells are treated with the following compounds: choleratoxin, PGE_1, isoproterenol, and dibutryl cyclic AMP. This is done in the presence of a labeled amino acid to label the newly synthesized collagen and other proteins. Following incubation the cultures are evaluated for rates of collagen and non-collagen protein synthesis as well as the concentration of cyclic AMP within the cells.

(6) To evaluate the post-translational modifications of the collagen molecule, cultures of fibroblasts from a variety of human sources are labeled with [^{14}C]proline or [^{14}C]lysine and the hydroxyproline in the newly synthesized collagen evaluated. Particular attention has been paid to the hydroxylation of the lysine residues and the subsequent glycosylation of the hydroxylysine residues with galactose or glucose-galactose. Following incubation, the newly synthesized collagen is isolated from the cultures and the relative amounts of hydroxylysine and glycosylated hydroxylysine are evaluated using an amino acid anlayzer.

(7) Collagen messenger RNA is being isolated from a variety of tissues abundant in cells which synthesize collagen. Most fruitful have been the use of the sheep and calf tendon and skin. The RNA is being isolated by phenol extraction or guanidine extraction. Following partial purification, the messenger RNAs are translated in a cell-free system derived from the rabbit reticulocyte. Purifications of collagen messenger RNA is being done with sucrose gradient methods and olgio-dT column chromatography.

(8) The destruction of collagen by collagenase has been evaluated in a number of systems including: the alveolar macrophage, human fetal lung fibroblasts, and the human neutrophil. Collagenase is assayed with [^{14}C]-labeled collagen fibrils or with labeled soluble triple helical collagen made in tissue

culture with human or rabbit lung fibroblasts. Collagenases are isolated primarily by culture techniques. Alveolar macrophages are cultured in a variety of conditions, the media collected and partially purified. Similar methodologies are used for fibroblast cultures. For the neutrophils, most studies have utilized the natural degranulation of the neutrophils during a 24-hour culture period. Other studies have utilized a specific isolation of neutrophils granules, either together or by purifying the azurophilic and specific granules.

(9) Methods have been developed in this laboratory to evaluate the possibility that a significant amount of collagen synthesized by cells is destroyed within the cell prior to its secretion. To do this we utilized [^{14}C]proline or [^{14}C]lysine to label the collagen being synthesized by explants of lung from rabbit or human lung fibroblasts. After varying incubation periods, the total amount of labeled hydroxyproline or hydroxylysine is quantitated before and after dialysis. The post-dialysis specimens are used to represent newly synthesized collagen larger than 10,000 daltons; i.e., that which has not been degraded. Similar methods are used to evaluate the time course of this destruction of collagen. A variety of controls are done including inhibitors of proteolysis and evaluation of cultures for more traditional mechanisms of collagen destruction such as collagenase.

(10) The past year we have been extensively purifying collagen Types I and III from calf and human sources to prepare antibodies that are type-specific. This purification has been complete and the antibodies prepared. The antibodies are being used for studies involving immunofluorescence evaluation of the localization of collagen Types I and III in cell culture (See Project No. Z01 HL 02403-04 PB) and lung biopsy from patients (See Project No. Z01 HL 02407-03 PB). In addition, these antibodies are being used for: (a) controls for evaluating patients in regards to circulating antibodies against collagen; and (b) to evaluate possible interactions of the humoral immune system directed against collagen on the surface of lung cells and how these mechanisms may change the differentiated state of these cells.

Major Findings: Studies in the past year have accomplished the following:

(1) Studies of the specific collagen types synthesized by cells of the lung have shown that AK-D, a tissue culture analog of the feline fetal epithelial cell (probably a Type I cell or a cell in transition from Type II to Type I), synthesizes both Type I and Type III collagen. HFL-1, a human fetal lung fibroblast, also synthesizes Type I and Type III collagen. Studies on the maintenance of the differentiated state with regards to the relative rigidity of the synthesis of Types I and III cells in culture have shown that NB-6, a newborn rabbit lung fibroblast, synthesizes approximately 65% Type I and 35% Type III. Evaluation of the relative amounts of these collagens synthesized at different times after subcultivation and at different subcultivation numbers have demonstrated that the same amount of collagen Types I and III are synthesized by the cell at all times. Thus, the differentiated state, with regard to specific types of collagen being synthesized, appears to be very well preserved for extended periods in culture.

(2) Detailed studies of the rates of collagen synthesis of HFL-1, a human fetal lung fibroblast, have demonstrated that it synthesizes the same relative amounts of collagen (relative to all the protein synthesized by the cell) over many population doublings. However, there appears to be a significant difference in the relative amount of collagen synthesized by these cells as to whether they are in the log or the confluent phase of cell growth. The rates of collagen synthesis is significantly higher in the confluent phase of growth compared to the log phase of growth.

(3) Evaluation of the rates of collagen synthesis by explants of human lung from patients with fibrotic lung disease have demonstrated that these patients' lung cells synthesize the same amounts of collagen per cell as does the normal. This finding has major implications for the understanding of the pathogenesis of idiopathic pulmonary fibrosis. It suggests that the disorder is not due to lung cells synthesizing excess amounts of collagen, but rather associated with lung cells synthesizing the normal amounts in the wrong places.

(4) Preliminary studies of cell-mediated immune control on the rates of collagen synthesis by lung cells suggest that lymphocytes produce materials which selectively reduce the rates of collagen synthesis by lung cells.

(5) Studies of hormonal controls of collagen synthesis have demonstrated that PGE_1, choleratoxin and isoproterenol all cause elevations rise in intracellular cyclic AMP with an associated selective decrease in the rates of collagen synthesis by cells. These changes appear to demonstrate a specific hormonal response since exposure of cells to propanalol (a specific inhibitor of the β-adrenergic receptor) blocks the effect of isoproterenol.

(6) Studies on the post-translational modifications of the newly synthesized collagen molecule have demonstrated that, at least in skin, newly synthesized collagen has more lysine residues that are hydroxylated and that more of these hydroxylysine residues are glycosylated compared to extracted skin collagen.

(7) Preliminary studies have demonstrated that collagen messenger RNA can be isolated in relative abundance from a number of tissues. This is translated with fidelity in the rabbit reticulocyte cell-free system.

(8) The alveolar macrophage secretes a collagenase that is in a latent inactive form. This same cell secretes a neutral protease which will activate this collagenase. In the process of activiation, the molecular weight of the collagenase is reduced from approximately 40,000 to 30,000 daltons. Likewise, fetal lung fibroblast collagenase is also in a latent form. It is not clear at this time how it is activated *in vivo* but we know that it can be activated *in vivo* by neutral proteases such as trypsin. While rabbit alveolar macrophage and human fibroblast collagenase will degrade collagen Types I and III at equal rates, human neutrophil collagenase will degrade Type I collagen at a rate 15 times that at which it attacks Type III collagen. In addition, human neutrophil collagenase appears to be localized to the specific granules.

(9) Detailed studies of lung explants have demonstrated that 30 to 40% of the newly synthesized collagen is degraded intracellularly. This occurs very rapidly, at least within 8 minutes of the time the isotope tracer is added to the cultures. Since the collagen molecule takes 7 to 8 minutes to synthesize and an additional 20 minutes to be secreted, this suggests the degradation is occurring intracellularly. Interestingly, the intracellular breakdown of collagen in these explants appears to be invarient with time; i.e., as the cultures are continued from up to periods of 48 hours, the amount of collagen destroyed is not changed. Several control studies have been done to demonstrate that this is not due to collagenase nor to proteases released during preparation of the explants or during the analytic procedures.

(10) Studies with type-specific antibodies against collagen Types I and III have shown that fetal lung fibroblasts synthesize both types. Studies are just beginning to evaluate human lung biopsies from patients with idiopathic pulmonary fibrosis compared to controls in regards to the anatomic location of these specific collagens. Studies are also beginning to evaluate these patients for circulating antibodies against collagen Types I and III.

Significance to Biochemical Research and Institute Program: These studies are a progression of studies that have been ongoing in this laboratory for the last several years to evaluate the control of the synthesis and degradation of the extracellular matrix of the lung. The findings in the past year have given us important insights into the mechanisms by which collagen in the alveolar interstitium is synthesized and degraded. Specifically:

(1) The evidence is accumulating that collagen synthesis by fibroblasts appears to be a constitutive process. That is, the fibroblasts seem to synthesize collagen in the same amounts and same types independent of a number of factors. They can only change the amounts of collagen they synthesize when specific external influences are added; i.e., hormonal and/or immune mechanisms.

(2) The evaluation of rates of collagen synthesis by human lung biopsies gave the surprising result that the rates of collagen synthesis (averaged over all cells) is not significantly different than normals. This has forced us to change our concepts of the pathogenesis of idiopathic pulmonary fibrosis in that we now regard it as a disease of derangement rather than a disease of excess collagen.

(3) Studies of immune and hormonal controls of collagen synthesis are the first examples of how cells may be regulated in terms of the amounts of extracellular matrix that they synthesize. The interesting aspects of these mechanisms is that the materials produced by lymphocytes appear to decrease the amounts of collagen synthesized by the cells and that the hormonal control appears to do the same. Whether or not they are working through similar mechanisms will have to await further study.

(4) The studies of the post-translational modifications of collagen in regards to lysine residues have interesting implications in terms of regulation of the

collagen molecule. There are two interpretations of these findings. First, fibroblasts in culture may have more abundant active enzymes necessary to modify these lysine residues. If so, this would suggest that fibroblasts could be regulated in terms of the ways in which they modify collagen. Secondly, these studies may imply that the modified lysine residues in newly synthesized collagen are then changed as the collagen molecules matures into the fibril. Further studies will be necessary to separate these possibilities.

(5) Isolation of messenger RNA for collagen is of fundamental importance in answering a number of questions as regard to the control of collagen synthesis. Most importantly, it will enable us to make a gene copy so that it can be used as a probe to quantitate the amounts of collagen messenger RNA in a variety of tissue culture situations. This should enable the documentation of the biochemical basis of a variety of hereditary disorders involving collagen synthesis such as Ehlers-Danlos Syndrome and osteogenesis imperfecta. In addition, it should allow investigation of fundamental questions such as how many genes there are for collagen, what are the relative number of genes for each of the collagen chains, and how are the expression of these genes regulated under a variety of hormonal and immune conditions.

(6) The finding that there is a collagenase specific for one collagen type is fundamental for the concept of an understanding of the regulation of the characteristics of the extracellular tissue. It has been previously thought that collagen quantities and types are regulated primarily by the synthetic mechansims. The finding that the neutrophil collagenase digests Type I collagen much faster than Type III allows the conceptulization of degradative mechanisms relative to the control of collagen regulation. The concepts of activation of latent collagenase by a neutral protease gives another level of control by which cells may modulate the extracellular matrix.

(7) The finding that significant portion of collagen is destroyed intracellularly is a novel concept in the collagen field. It gives another level of control, perhaps by which cells may destroy abnormal forms of collagen prior to these collagens getting out into the extracellular space.

Proposed Course to Project: Our studies of the control of synthesis and degradation of the extracellular matrix will continue as we culture more cell types from animal and human lung. The studies on the types of collagen being synthesized will be continued. As technology develops to evaluate new kinds of collagen (i.e., basement membrane) these will also be evaluated.

Fundamental to our understanding of the structure and function of lung in health and disease is an understanding of how cells maintain their differentiated state. Since connective tissue is so fundamental to the maintenance of structure and function we will continue to evaluate how cells regulate the connective tissue they synthesize and degrade. The studies will be expanded in terms of hormonal, immune, and inflammatory modulation of the maintenance of the differentiated state by lung cells.

The molecular biologic approaches to an understanding of collagen regulation will be expanded and can be applied to the understanding of the maintenance of the differentiated state using several of the approaches discussed above.

We will continue studies in understanding the processes by which collagen is degraded by cells of the lung. Of particular interest is the modulation of intracellular breakdown in normal conditions and possibly as a regulatory mechanism by hormonal, immune, and inflammatory processes. It is also possible that some of the hereditary disorders of connective tissues such as osteogenesis imperfecta may be involved with increased intracellular degradation of one collagen type relative to another.

Publications:

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Horwitz, A.L. and Crystal, R.G. Collagenase from Rabbit Pulmonary Alveolar Macrophages. Biochem. Biophys. Res. Comm., 69, 296-303, 1976.

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Horwitz, A.L., Kelman, J.A., and Crystal, R.G. Activation of Alveolar Macrophage Collagenase by a Neutral Protease Secreted by the Same Cell. Nature, 264, 772-774, 1976.

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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Composition of the Interstitial Extracellular Matrix

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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0.6	0.6	

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)

The primary objective of these studies is to identify the heterogeneous types of collagen in lung parenchyma. Studies have shown that the interstitial collagens of the parenchyma are composed of two types: Type I and Type III. Type I is the thick, fibrillar that is noncompliant. Type III collagen are the loose, randomly arranged fibrils that are probably more compliant. Studies are ongoing to quantitate these collagen types in the parenchyma of normal animal and human lung in health and disease. Since the mechanical properties of lung are critically dependent on the types of collagen comprising it, the heterogenetiy of lung collagen is a critcally important determinant of lung function.

1155

Project Description:

Objectives: Collagen is the most abundant protein in lung comprising 10 to 20% of the adult lung by dry weight. There are more than 40 cell types in lung, including mesenchymal cells (fibroblasts), epithelial cells, endothelial cells, and smooth muscle cells, all of which have been implicated to synthesize collagen. The primary objective of these studies is to identify the heterogeneous types of collagen in lung. In combination with our studies on the synthesis and degradation of the extracellular matrix (See Project No. Z01 HL 02409-02 PB), we hope to be able to determine the cells responsible for the synthesis and degradation of this material.

Prior studies have demonstrated that the lung parenchyma of animal and human lung is composed of at least two types of collagen: Type I collagen $[\alpha 1(I)]_2\alpha 2$ and Type III collagen $[(\alpha 1)III)]_3$. Because the lung has morphological evidence of extensive basement membrane it is likely that basement membrane collagen is present in lung parenchyma as well. However, at present, it is not clear whether this material is composed of a single form of collagen or a multiple species. In either case, it probably represents less than 10% of the total interstitial collagen. The lung also contains Type II collagen (cartilage collagen) in the tracheo-bronchial tree. The tracheo-bronchial tree is also surrounded by small amounts of Type I and Type III collagen. The major vessels in the lung also contain Type I and Type III collagen.

The interest in collagens Type I and III revolve primarily around the general concept that they have different morphological and probably mechanical properties. In vitro studies suggest that Type I collagen is the thick, cross-banded fibrils that are stiff and noncompliant. In comparison, Type III collagen is the loose, randomly arrayed fibrils that used to be termed reticulin. Although the evidence is indirect, it is more likely that Type III collagen is more compliant than Type I collagen. Thus, the relative amounts of Type I and Type III collagen within the alveolar interstitium probably have profound effects on the mechanical properties of the lung.

The purpose of these studies is to establish the quantities of Type I and Type III collagen in lung parenchyma in both health and disease. Two approaches are being used: (1) evaluation in the changes in ratio of Type I and Type III collagens with lung growth and development; and (2) evaluation in the changes of parenchymal lung collagen Types I and III in health and disease. In the latter, we have concentrated primarily on evaluating the fibrotic lung disorders.

Methods: The primary methods used for evaluating the types of collagen and quantitating the amounts present is by using cyanogen bromide peptide mapping techniques. This involves combinations of carboxymethyl cellulose chromatography, sodium dodecyl sulfate acrylamide gels, and isoelectric focusing gels. Because there are 5 to 10 methionine residues per 1,000 amino acid residues in collagen α chain, cyanogen bromide is particularly suitable for cleaving collagen (it cleaves only in methionine residues);

leaving peptides which are few enough in number to be able to handle. Although collagen is very difficult to extract from intact lung, cyanogen bromide cleavage of the entire lung parenchyma has enabled the evaluation of the types of collagen present.

Similar methods have been developed to quantitate and identify the types of collagen synthesized by explants of lung and tissue culture systems (See Project No. Z01 HL 02409-02 PB).

Major Findings: Studies have clearly identified that both collagen Types I and III are present in the rabbit and human lung parenchyma. There are still a number of methodologic problems to be overcome to enable the accurate quantitation of these types. Some of these problems regard the probable differences in the amino acid sequences of rabbit and human Type III collagen. As a result, the peptide patterns (particularly in a region termed the α1(III) CNBr 3 and 4 region) are very complex. Work is ongoing to clarify this problem in order to proceed with the quantitative work.

In parallel, antibodies have been prepared that are monospecific against Type I and Type III collagen. These are being used to establish the anatomic distribution of collagen Types I and III in both normal and diseased human lung.

Significance to Biochemical Research and Institute Program: Normal lung depends on the collagen comprising it. Since the mechanical properties of lung are critically dependent on the types of collagen comprising it, the heterogeneity of lung collagen is a critically important determinant of how the lung functions in health and disease. During the developmental process there are major changes in the control of collagen synthesis and in the relative amounts of collagen within the lung and probably the types of collagen present. An understanding of these processes will help toward the understanding of the pathological processes in diseased lung.

The fibrotic lung diseases have many primary stimuli including the inhalation of toxic materials, hypersensitivity states, and radiation injury. In addition, pulmonary fibrosis is often associated with systemic diseases such as progressive systemic sclerosis. It is likely that these disorders are associated with injury to different lung cells, suggesting the possibility that there may be a heterogeneity in the types and/or amounts of collagens present in these different lung disorders on the basis of types of collagen synthesized in a similar fashion to the lipoprotein and hemoglobin disorders.

Proposed Course to Project: The major areas to complete are in developing the methods to quantitate Types I and III collagen in the parenchyma in rabbit and human lung. This will be correlated with immunofluorescent studies using monospecific collagen antibodies. If shifts in the types of collagen present in the interstitium in growth and development and/or in lung disease are found, attempts will be made to determine the mechanisms by which this occurs.

Publications:

Hance, A.J., Bradley, K. and Crystal, R.G. Lung Collagen Heterogeneity. Synthesis of Type I and Type III Collagen by Rabbit and Human Lung Cells in Culture. J. Clin. Invest., 57, 102-111, 1976.

Hance, A.J., Horwitz, A.L., Cowan, M.J., Elson, N.A., Collins, J.F., Bienkowski, R.S., Bradley, K.H., McConnell-Breul, S., Wagner, W.M., and Crystal, R.G. Biochemical Approaches to the Investigation of Fibrotic Lung Disease. Chest, 69 (Suppl.), 257-261, 1976.

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Hance, A.J. and Crystal, R.G. Collagen. In: R.G. Crystal (ed.). The Biochemical Basis of Pulmonary Function, M. Dekker, New York, 215-271, 1976.

PROJECT NUMBER (Do NOT use this space)	HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	Z01 HL 02411-01 PB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Control of Cell-Cell and Cell-Matrix Interactions

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

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	D. M. Rosenberg	Clinical Associate	PB NHLBI
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COOPERATING UNITS (if any)

V. Ferrans, Pathology Branch, NHLBI

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Pulmonary Branch

SECTION

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NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.9	2.6	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)

The major cell surface protein of cultured fibroblasts is a large (220,000 dalton) glycoprotein termed CSP. There is a growing body of evidence that CSP is one of the biological macromolecules responsible for orderly cell-cell and cell-substratum interactions and for the maintenance of normal cell morphology. Human lung fibroblasts shed CSP from the cell surface with a half-life of 25 hours. Simultaneous with the loss of CSP from the cell surface, a similar macromolecule appears in the culture medium. This shedding process appears to be stoichiometric. Studies with human neutrophil proteases have demonstrated that human fibroblasts CSP is remarkably sensitive to proteolysis. The small amounts of these proteases will destroy the ability of CSP to agglutinate cells and to attach cells to a collagen substrate. These studies suggest that one of the effects of the inflammatory process is to destroy macromolecules such as CSP such that cellular order is disrupted.

1059

Project Description:

Objectives: The major cell surface protein of cultured fibroblasts is a large (220,000 dalton) glycoprotein. This macromolecule or closely related form has been studied under a variety of names including cell surface protein (CSP), large external transformation sensitive protein, fibroblast surface antigen, and fibronectin. There is a growing body of evidence that CSP is one of the biological macromolecules responsible for orderly cell-cell and cell-substratum interaction and for the maintenance of normal cell morphology. Current evidence suggests that CSP interacts with the extracellular matrix by associating with a specific site on the collagen molecule. In fact, there may be cell specific CSP's that are critical for determining which cells will stick to which matrices.

Another interesting aspect of CSP is that a protein known to be a major component of serum (termed cold insoluable globulin or CIG) has similar, if not identical, characteristics to CSP. It is possible that CIG represents the CSP that has left the surfaces of cells and entered the circulation. This is of particular interest because CIG might be used as a serum marker for the relative status of cell-cell and cell-matrix interactions in the body, particularly if they turn out to be cell specificities for these groups of macromolecules.

Thus, CSP respresents a handle by which it is possible to begin investigations into the biological control of how cells interact with one another and to their extracellular matrix. Importantly, this system can be used to evaluate those effector processes which modulate these interactions.

The purpose of these investigations are to: (1) determine the processes by which CSP leaves the surface of normal human fibroblasts; (2) to evaluate ways in which the inflammatory system may interact with CSP to disrupt cell-cell and cell-substratum interactions; and (3) evaluate the influence of CSP on the ordering of the extracellular matrix itself.

Methods: All studies are being done with a diploid fibroblast strain (HFL-1), derived from the parenchyma of a 16-week-old human fetal lung.

To evaluate the production of CSP by normal human lung fibroblasts, confluent cells were incubated in media containing [^{14}C]proline, [^{14}C]glucosamine, or [^{3}H]fucose. In addition, the protein on the surface of HFL-1 cells were labeled using a lactoperoxidase catalyzed reaction with ^{125}I and the cell surface proteins extracted with urea. The cells and the media were analyzed separately by displaying the labeled proteins on sodium dodecylsuflate polyacrylamide gels with audioradiographic techniques.

To evaluate the functional characteristics of human lung fibroblast CSP, two assays were used: (1) agglutination of formalin treated sheep red blood cells (cell surface protein has been shown to modulate the adhesion of the cells treated in such a manner); and (2) the adhesion of Chinese hamster ovary cells to a collagen substrate in a tissue culture plate.

To evaluate the possible interactions of the inflammatory system with human lung fibroblast CSP, human neutrophil granules were isolated and neutrophil proteases recovered. The activities of these proteases were tested against a [^{14}C]-globulin substrate. The effect of these proteases on cell-cell and substratum interactions were then tested using the agglutination and cell adhesion assay described above. To evaluate the direct effect of proteases on cell surface protein, ^{125}I-labeled CSP was purified from HFL-1 cells and incubated in vitro with human neutrophil protease. To demonstrate morphologically the effect of proteases on the cell surface protein, an antibody was prepared in goats against purified human cold insoluble globulin (which cross-reacts antigenically with human CSP). Using an indirect immunofluorescent technique the presence of CSP on cell surfaces and the effect of proteases on this immunofluorescent pattern was evaluated.

To evaluate the interactions of CSP and collagen an in vitro method was developed. Collagen fibers were formed on glass slides in the presence or absence of CSP. The morphological form of both the collagen and the CSP were then evaluated using indirect immunofluorescent methods with antibodies against CIG and antibodies against specific collagen types.

Major Findings: With time, human lung fibroblast CSP is progressively lost from the cell surface with a half-life of approximately 25 hours. Simultaneous with the loss of CSP from the cell surface, a similar macromolecule appears in the culture medium. Several lines of evidence suggest the soluble medium protein is derived from CSP: (1) it has a molecular weight of about 220,000 daltons; (2) as with CSP, this medium protein is a glycoprotein; (3) when the cell surface proteins of HFL-1 cells were iodinated and the cells placed in fresh medium, an ^{125}I-labeled protein of 220,000 daltons appeared in the medium; and (4) the labeled 220,000 dalton glycoprotein in the medium cross-reacts with an antisera prepared against CSP. Thus, normal human lung fibroblasts shed the major cell surface glycoprotein continually from the cell. Most importantly, this shedding process appears to be stoichiometric; that is, there is a one-to-one relationship between the 220,000 dalton glycoprotein appearing in the media and the CSP lost from the cell surface. This is of particular importance in relation to the finding that cold insoluble globulin in serum cross-reacts antigenically with cell surface protein.

The 220,000 dalton media glycoprotein of normal human cultured fibroblasts is probably the culture analog of the intermediate step of shedding of this protein from the cell surface and its eventual release into the circulation. Since CSP is critical for cell-cell and cell-matrix interactions, it is possible that cold insoluble globulin in patients with various disorders may reflect the general state of order of cells in a specific organ.

Studies with human neutrophil proteases have demonstrated that human fibroblast CSP is remarkably sensitive to proteolysis. If small amounts of human neutrophil proteases are placed on human lung fibroblasts in culture, the cells rapidly lose their CSP on their surfaces as demonstrated by immunofluorescence. In addition, when a purified preparation of CSP is briefly exposed to human

neutrophil proteases, the preparation loses its ability to agglutinate formalin treated sheep red blood cells. In addition, the preparation of CSP loses its ability to attach the Chinese ovary cells to a collagen substrate. These studies suggest that one of the effects of the imflammatory process is to destroy macromolecules such as CSP such that cellular order is disrupted. This has major implications in relation to inflammatory disorders not only in lung but other diseases as well.

Preliminary studies have also suggested that antibodies against CSP will be useful in understanding the interactions of CSP with specific collagen types; particularly in understanding the self-aggregation properties of these macromolecules.

Significance to Biochemical Research and Institute Program: A number of chronic lung disorders involve inflammation of the lung parenchyma. The definition of a macromolecule that is important in maintaining cell order is a first step in beginning to understand how the lung maintains its normal structure. Since these cell-cell and cell-matrix interactions can be interrupted by proteases released from neutrophils, these findings suggest that one of the ways that the inflammatory system can destruct the pulmonary parenchyma is by destroying those macromolecules that interact between cells and the extracellular matrix. If it turns out that there are specificities of these macromolecules (i.e., for specific cell types) this will have major implications in terms of understanding how the lung assembles itself in a normal fashion and why in certain disease states the lung cannot effect normal repair. If it turns out that cold insoluble globulin is a serum marker for CSP, these studies may yield a clinical tool to evaluate order-disorder in the interstitium of the lung. As a clue that this may be the case, we know that 50 to 60 percent of patients with idiopathic pulmonary fibrosis have elevated levels of cryoglobulins. Since studies from other laboratories have suggested that cold insoluble globulin can be associated with cryoglobulins, the elevated levels of cryoglobulins may reflect CSP shed from cells of the disordered alveolar interstitium.

Proposed Course to Project: These studies are being extended to: (1) use antibodies against CSP to evaluate biopsy material from patients with a variety of inflammatory lung disorders; (2) determine the mechanisms by which CSP is released from the surface of normal cells and how this may be disrupted in disease; (3) evaluate fibroblasts cultured from the lung parenchyma of patients with a variety of lung disorders to determine whether or not these fibroblasts make these macromolecules in normal amounts, form and function; (4) continue studies on the interactions of cell surface protein and specific collagen types found in the lung; and (5) continue the investigation of the interactions of the inflammatory system in disrupting cell-cell and cell-matrix interactions.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HLB 02501-03 PB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Synthesis, Uptake and Storage of Biogenic Amines in Rat Lung

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

PI:	R. E. Shaff	Chemist	PB NHLBI
OTHER:	M. A. Beaven	Pharmacologist	PB NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Pulmonary

SECTION
Molecular Pharmacology

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NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.1	0.4

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Slices of rat, rabbit and guinea pig lung were shown to take up labeled serotonin by an active process that was reminiscent to that observed by other authors in intact lung preparations. The uptake was blocked by Lilly 110140, a compound known to block serotonin transport in neurones and platelets. Since the labeled serotonin taken up by the slice was not released by reserpine as was endogenous serotonin, it was concluded that the label had not entered endogenous stores. Further studies suggested that the uptake and serotonin metabolism were coupled processes. When lung slices were incubated in the presence of an MAO inhibitor, labeled serotonin accumulated within the tissue. In the absence of the inhibitor, serotonin did not accumulate and was rapidly converted to hydroxyindole acetic acid, which diffused back into the medium. In the presence of Lilly 110140, uptake and metabolism were inhibited. Lilly 110140 did not inhibit MAO activity in lung homogenates in concentrations that inhibited uptake and metabolism in the intact slice.

1163

Introduction: A number of studies in vivo have shown that lung actively takes up serotonin from the circulation by an energy-dependent process. We have previously shown that this uptake can be reproduced in vitro with lung slices from rat (see Annual Report Z01 HLB 02501-02 PB, 1976). The studies indicated that serotonin, but not histamine, is taken up by a mechanism with the characteristics of active transport. The uptake is inhibited by metabolic poisons such as sodium cyanide, ouabain, dinitrophenol, by low temperature (0°C) and by compounds known to block transport of serotonin into neurones and platelets as, for example, imipramine and the Lilly compound 110140. The uptake is saturable ($K_m \sim 5 \times 10^{-7}$), rapid ($t_{1/2} \sim 5$ min) and relatively specific. Tyramine and, to a lesser extent, tryptamine compete for the uptake, but N-acetylserotonin, 5,6-dihydroxytryptamine, norepinephrine do not. Since destruction of serotonergic and adrenergic neurones by 5-hydroxydopamine and 5,6-dihydroxytryptamine did not affect uptake, the stores were non-neuronal. As indicated by histological fluorescence studies, serotonin is present in mast cells located exclusively around small blood vessels. The present studies were undertaken to see if the serotonin taken up by the slice was incorporated into endogenous stores. The uptake mechanism in other species was also studied.

Methods: Rat, guinea pig and rabbit lung slices (∿ 100 mg) were prepared with a Stadie-Riggs microtome and incubated in 2 ml Tyrode solution at 37° in flat-bottomed flasks. A monoamine oxidase inhibitor (pargyline) was included in the mixture to prevent metabolic destruction of the serotonin. Drugs and labeled histamine or serotonin were added to the medium and aliquots of the medium removed at appropriate intervals for radioassay of the amines and their metabolites as described in previous reports. Release of labeled amines was studied by preincubating the slice with labeled amine for 30 min. The slice was briefly washed in Tyrode Ringer and transferred to fresh medium.

Results: 1) Studies of Uptake of Serotonin with Lung from other Species. As with rat lung slices, [^{3}H]-serotonin was actively taken up in vitro by lung slices obtained from guinea pig and rabbit. Uptake was evident at 37°C but not at 4°C. Concentratioon ratios (medium to slice) > 1:10 were observed with lung from all three species. As observed with rat lung, the uptake was blocked by the serotonin transport inhibitor, Lilly compound No. 110140, in concentrations of 10^{-5} M.

2) Studies with Reserpine. To determine if the labeled serotonin had equilibrated with endogenous serotonin stores in the mast cells, studies were undertaken to see if endogenous and labeled amine were released in equivalent proportions from slung slices prelabeled with ^{3}H-serotonin. Our earlier studies in vivo had shown that the serotonin stores in rat lung were resistant to the depleting action of compound 48/80 but were partially depleted by reserpine. We found that the endogenous stores could be similarly depleted in vitro by reserpine. Compared with control incubations (adjacent slices of lung incubated without drug), 31-73% of the endogenous amines was released. However, in slices that had been preequilibrated for 30 min with ^{3}H-serotonin and then washed, release of label in the presence of reserpine was slight (29% in one experiment) or insignificant (< 10%, n = 15). It was concluded from

this study that the label serotonin had not equilibrated with the endogenous serotonin.

3) Possible Coupling of Serotonin "Uptake" and Metabolic Inactivation in Lung. Because of the above result, alternative explanations for the uptake process were sought. Our studies indicated that in the absence of MAO inhibitors uptake was not apparent. Instead, the labeled serotonin was rapidly metabolized to 5-hydroxyindole acetic acid, which diffused into the medium. This rapid destruction raised the question of whether the uptake of serotonin by lung was coupled to metabolic destruction. Almost all (> 90%) of the MAO activity in rat lung is intracellular and particulate (Beaven and Shaff, unpublished data), and mechanisms must exist to allow free passage of the amine onto intracellular enzymes.

In the presence of different concentrations of Lilly 110140 (10^{-4}-10^{-6} M) and pargyline, the uptake of serotonin was inhibited to varying degrees in a dose-dependent fashion. When pargyline was omitted, the conversion of serotonin to 5-hydroxyindole acetic acid was also inhibited by Lilly 110140. At 10^{-4} M, a concentration which was sufficient to inhibit uptake completely, metabolism of serotonin by the slice was minimal (< 15%). Inhibition of serotonin metabolism was not observed with Lilly 110140 in lung homogenates at this concentration.

General Conclusions and Proposed Course of Work: Our previous studies indicated that histamine and serotonin exist in separate stores in the rat lung and that serotonin is stored exclusively in mast cells around small blood vessels. It was suggested that serotonin may have a role in the lung vasculature (see last year's annual report). The present studies indicate that the active uptake of serotonin by lung is not associated with serotonin stores in the mast cell but is coupled to the metabolic degradation of serotonin. The uptake mechanism may therefore act as a scavenger to remove free serotonin from the circulation.

Further studies will be designed to answer the question of whether serotonin, like histamine, is released by antigen-IgE reactions from rat lung and whether serotonin is stored in mast cells along the vasculature in lung of other species.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HLB 02503-05 PB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Histamine Release and Metabolism in Allergic and Inflammatory Reactions

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

PI:	Z. Horakova	Pharmacologist	PB NHLBI
OTHER:	M. A. Beaven	Pharmacologist	PB NHLBI

COOPERATING UNITS (if any)
Dr. Allen Kaplan, Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases; Dr. Stephen Katz, Dermatology Branch, NCI; Dr. L. M. Lichtenstein, Department of Medicine, Johns Hopkins University, Baltimore, Maryland

LAB/BRANCH
Pulmonary

SECTION
Molecular Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	0.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A survey of histamine levels in plasma, urine and blood in patients with various disorders indicates that the appearance of histamine in plasma is found only in patients with severe mastocytosis, chronic myelogenous leukemia, anaphylactic reactions and during severe urticarial reactions in certain forms of physically induced urticarias. All these patients had symptoms attributable to histamine release, e.g. increased gastric secretion, hypotension. Elevated blood histamine was observed in all cases of chronic myelogenous leukemia and polycythemia vera. Urine histamine was also elevated in these disorders, even in the absence of histamine-like symptoms. Urine histamine measurements were found to be especially useful in distinguishing patients with cutaneous mastocytosis from those with systemic mastocytosis.

1166

Introduction: An important objective of this section is the development of new approaches and methods in the diagnosis and study of diseases which may involve disorders in the release and metabolism of the biogenic amines. The discovery of high histaminase (diamine oxidase) activity in medullary thyroid carcinoma (N.Engl.J.Med. 283: 1239, 1970) led to extensive evaluation of the usefulness of the enzyme as a marker for this tumor (Lancet I: 455, 1972; Am.J.Med. 53: 723, 1972: Ann.Int.Med. 78: 561, 1973; N.Engl.J.Med. 289: 545, 1973). Another project has been the assessment of the utility of the isotopic enzymatic assay of histamine in the diagnosis of diseases with known abnormalities of histamine production and release. The main activity this year has been the completion of the program and continued investigation of histamine release in patients with various forms of physically induced urticarias.

Methods: The various procedures have been described in detail in previous reports and were summarized in last year's annual report.

Results: Urine and blood histamine levels in various disease states. Measurements have been completed in over 100 subjects and patients. Comparison of our data with previously published data on histamine levels in blood and urine are summarized in Table 1. The values obtained for blood histamine are comparable to those obtained by other workers with the fluorometric and biological procedures. The values for urine are comparable to those obtained by biological assay but are lower than those obtained by fluorometric assay. The values of the latter procedure are known to be spuriously high due to interference of spermidine and NH_3.

The values obtained for urine in the various disease states are summarized in Table 2. Elevated histamine levels were observed in patients with mastocytosis and the basophilic disorders. Elevated histamine levels were also observed during a severe anaphylactic reaction. Of specific interest was that there appeared to be a clear distinction in the urine histamine levels in patients with systemic and those with cutaneous mastocytosis. The data point to the possible usefulness of such measurements in distinguishing between these two forms of mastocytosis.

Histamine was not detectable (< 1 ng/ml) in plasma of normal subjects and most patients. The few patients in which measurable levels have been observed include 3 patients with mastocytosis and one with CML. All these patients had hyperchlorhydria. Transient increases in plasma histamine have been noted in patients with urticarial reactions as noted below.

In whole blood, histamine levels were elevated only in subjects with the basophilic leukemias. In all other diseases, including mastocytois, the levels remained normal.

Origin of urinary histamine. Studies with histidine loading. The tissue mast cell and blood basophil are clearly a source of urinary histamine in patients with mastocytosis and basophilia. Studies with L-histidine suggest that the kidney can also be a source of urine histamine. Large increases in

urine histamine were observed upon loading with L-histidine. The extent of increase was related to dose of the amino acids, and with the highest dose (48.6 and 64.8 g), urine histamine levels exceeded those seen in any of the disorders mentioned above. Despite these high histamine levels, there were no increases in blood or plasma histamine levels and no symptoms associated with circulating histamine, either in normal subjects or in a patient with mastocytosis. One possible explanation for these results is that histamine was formed locally in the kidney, which has a high decarboxylase activity, and excreted directly into the urine.

Further studies in patients with allergic- and physically induced urticarial reactions. Elevated plasma histamine levels have been found in the venous drainage after induction of a urticarial reponse in disorders such as cold- and exercise-induced urticaria (see last year's report). In order to examine mediator release at the site of urticarial lesions, blisters were induced over lesions and normal skin sites by suction and mild heat (45-52°C). The blister fluid was then aspirated and assayed. The blister fluid from normal controls had 6.6 $\pm$ 1.2 ng/ml histamine while patients with cold urticaria had 9.5 $\pm$ 3.0 ng/ml histamine at normal skin sites and from 13-127 ng/ml histamine over the site of a lesion induced by application of an ice cube. Elevated blister fluid histamine was also demonstrable in patients with solar urticaria, immediate and delayed pressure urticaria, and local heat urticaria. Patients with chronic idiopathic urticaria with either vasculitis or perivascular infiltrate had histamine in lesional sites of 22-48 ng/ml while control sites free of visible urticaria had 48-93 ng/ml histamine. These patients therefore have either an elevated interstitial fluid histamine in areas free of lesions or have increased lability of mast cells during blister formation as well as lesional sites that are relatively depleted of histamine. No elevation of blister fluid histamine was demonstrable in patients with cholinergic urticaria.

Mediator release in human anaphylaxis. In one study by Lichtenstein and associates, three groups of patients who were sensitized to insect stings were given placebo, whole insect extract (WBE), or venom injection. The patients were continuously monitored and were challenged with increasing doses of venom. Systemic reactions (generalized urticaria) were noted in 7/11 WBE and 1/19 venom-treated patients. Of these, three developed hypotension. Changes in pulmonary mechanics were noted in one of these patients, although all three had a fall in PO_2. Histamine measurements by us indicated that blood histamine levels in patients with urticaria ranged from 0 to 6 ng/ml and were not correlated with the severity of the reaction. In the three patients with hypotension, peak histamine levels were 13, 60 and 145 ng/ml and paralleled the severity of the hypotension. In the latter instance, blood histamine levels were elevated for 40-50 min after challenge. In the two patients with most severe hypotension, there was a 5- to 10-fold diminution of factor V, factor VIII and fibrinogen as well as diminished high molecular weight kininogen. In one of these patients, there was also diminished levels of C4 and C3. In the mildest hypotensive patient as well as patients with urticaria, no significant changes in the coagulation or complement system were evident. It was concluded that in severe episodes of anaphylactic

shock, blood histamine levels are elevated and, in some instances, complement and/or kinin generating systems are activated.

Significance to Biomedical Research: The studies have defined the pathological conditions under which abnormal histamine levels appear in blood plasma and urine. They have further shown that in anaphylactic phenomena, and some forms of physically induced urticarias, histamine release is closely associated with development of symptoms. The studies also point to the possible utility of urine histamine measurements in distinguishing between systemic and cutaneous mastocytosis.

Publications:

Horakova, Z., Keiser, H.R. and Beaven, M.A.: Blood and urine histamine levels in normal and pathological states as measured by a radiochemical assay. Clin.Chim.Acta, in press.

Metzger, W.J., Kaplan, A.P., Beaven, M.A., Irons, J.S. and Patterson, R.: Hereditary vibratory angioedema: Confirmation of histamine release in a type of physical hypersensitivity. J.AllergyClin.Immunol. 57: 605-608, 1976.

Wilcox, G. and Beaven, M.A.: A sensitive and specific tritium assay for dopamine-β-hydroxylase (DβH) in serum. Anal.Biochem. 75: 484-497, 1976.

TABLE 1

Urine and Blood Histamine Levels in Normal Subjects as Determined by Different Assays

Study (reference)	Procedure	No. of Obs.	Histamine	
			Mean	(Range)
URINE			µg/24 hr	
Pearce and Valentine, 1950	Bioassay	25	27	(6-68)*
Mitchell and Code, 1954	Bioassay	5	15	(7-21)
Mitchell, 1956	Bioassay	6	15	(7-29)
Dunér et al., 1961	Bioassay	4	12	(6-19)
Granerus, 1968	Bioassay	20	23	(6-46)
Oates et al., 1962	Fluorometric	24	45	(15-90)
Gilbert et al., 1966	Fluorometric	19	42	(18-67)
Beaven et al., 1972	Enzymatic Assay	16	16	(< 5-92)*
Present study	Enzymatic Assay	31	19	(< 5-42)
BLOOD			ng/ml	
Haworth and MacDonald, 1957	Bioassay	103	40	(18-78)
Rose and Browne, 1940	Bioassay	60	40	(25-80)
Valentine et al., 1950	Bioassay	33	80	(35-140)
Zachariae, 1963	Fluorometric	32	100	(40-140)
Gilbert et al., 1966	Fluorometric	67	40	(10-100)
Present study	Enzymatic Assay	11	56	(11-105)

* All except 1 subject in each study excreted less than 45 µg/24 hr.

TABLE 2

Histamine Content of Body Fluids under Normal and Pathological Conditions[4]

		Urine (g/24 hr)		Whole Blood (ng/ml)		Plasma (ng/ml)	
	n	mean	range	mean	range	mean	range
1) Normal male	19	19	< 5-42	56	12-105	< 1	--
female	12	18	< 5-92				
children (< 12 yr)	6	8	< 5-20	--	--	< 1	--
2) Mastocytosis systemic	5	209	150-413	77	--	2	< 1-8
cutaneous	7	68	39-88	33	--	< 1	--
3) Basophilic leukemias							
chronic myelogenous leukemia	6	861	323-1060	10,800	450-27,000	1	< 1-11
polycythemia vera	5	48	84-152	388	101-602	--	--
4) Anaphylactic reaction (yellow jacket stings)	4	59 µg*	--	--	--	--	13-145
5) Cold-induced urticarias	8	--	--	--	--	--	8-40
6) Miscellaneous diseases (Migraine, Oates cell and medullary carcinoma, Dengue's Fever, etc.)	22	16	< 5-88	--	--	--	--

* 12-hr period--one patient.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HLB 02513-05 PB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Purification and Characterization of $Na^+ + K^+$-ATPase

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

PI:	T. N. Lo	Visiting Fellow	PB NHLBI
OTHER:	E. O. Titus	Chief, Section on Molecular Pharmacology	PB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Pulmonary

SECTION
Molecular Pharmacology

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NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	1.3	0.3

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The rates and position of cleavage by trypsin of the larger of two proteins in $(Na^+ + K^+)$-ATPase, an enzyme responsible for cation transport in cell membranes, are dependent on ion and ATP-induced conformational changes. Occupancy of protein sulfhydryl groups by N-ethylmaleimide blocks the ATP effect. The smaller glycoprotein, normally resistant to trypsin, is rapidly hydrolyzed when the enzyme is in a catalytically inactive conformation induced by potassium, magnesium and ATP.

1172

Project Description:

Objectives: The sodium plus potassium-dependent ATPase is the active transport system that moves monovalent cations across cell membranes against electrical and chemical gradients and thus maintains the excitability of nerve and muscle. It is of interest as the receptor for digitalis glycosides and is a model for the study of information transfer across cell membranes, since extracellular glycosides regulate the intracellular catalytic site. The goals of this project are to describe the molecular events (presumably reversible changes in conformation of the ATPase) that are associated with ion transport and to ascertain the major structural features of the system.

The enzyme, as isolated from several membranes, is a particulate complex of lipids and two proteins. Our earlier studies have shown that the larger of these proteins traverses the whole membrane and undergoes major conformational changes as the transport enzyme is phosphorylated and dephosphorylated during ion movement. Certain of these conformational changes protect and others expose protein sulfhydryl groups to reaction with radioactive labeling reagents such as N-ethylmaleimide. Current research is directed toward the location of these conformationally sensitive sites as a first step in structural studies of the enzyme.

Methods Employed: Purified ATPase is prepared from rabbit kidney microsomal fragments by modification of Jørgensen's (Methods in Enzymology 32: 277, 1974) procedure. Certain of the conformational states through which the enzyme must pass during ion transport are stabilized by adding ionic ligands or by ion-induced phosphorylation with ATP. Concentrations of available sulfhydryl groups are determined by labeling with ^{3}H- or ^{14}C-N-ethylmaleimide (NEM). Conventional analytical disc-gel electrophoresis of proteins solubilized in sodium dodecyl sulfate is used to characterize the ATPase and the hydrolytic products obtained by exposure to trypsin or various conformational states.

Major Findings: It was shown earlier that ligand-dependent conformational changes in the major protein of transport ATPase are sensed by trypsin. In the presence of potassium ion trypsinolysis is initiated near the center of this 96,000 dalton protein. With sodium and especially with ATP the point of initial cleavage is shifted toward one end of the molecule, since fragments of 75,000 daltons accumulate. All ligands slow the rate of hydrolysis. The small chain is resistant to hydrolysis.

Current studies were undertaken in the hope that SH groups essential for catalytic activity could be labeled by NEM and at least partially localized along the major chain by measuring the distribution of label in the larger fragments from proteolysis. Prior reaction with NEM markedly increased the rate of attack by trypsin and reversed the effects of ATP on the point at which cleavage was initiated. The evanescent appearance of fragments of 50,000 daltons indicated that the cleavage of NEM-treated enzyme again occurred near the middle. The temporary accumulation of proteolytic products with molecular weights of 30-50 thousand, a phenomenon not seen with control enzyme, was observed with the NEM enzyme.

The effects of other ligands on trypsinolysis of ATPase were examined. Concanavalin A, a bulky inhibitor that would be expected to bind to extracellularly oriented sugars on the small glycoprotein component of the enzyme was tested in the hope that blockade of the ATP-induced effect might indicate that one cleavage point was on the extracellular end. Con A was without effect, with or without ATP.

Oligomycin, which inhibits the enzyme by blocking conformational transitions in a phosphorylated intermediate stage but which does not block partial reactions catalyzed independently by sodium or potassium, caused some evanescent accumulation of breakdown products of intermediate molecular weight. It has been shown that a complex of ATPase with magnesium, potassium and ATP is catalytically inactive and relatively stable, since there is a significant delay in return to active status after the addition of activating ions. The tryptic hydrolysis of ATPase, normally slowed by ATP or potassium salts alone, is enhanced by this complex formation. More importantly, the hydrolysis of the smaller glycoprotein, which is normally negligible until most of the larger protein has disappeared, is now rapid. Inactivation is thus accompanied by some reorientation of the two proteins in the matrix.

In the presence of ATP alone, under which conditions one mole of ATP is bound to one unit of active enzyme (an assembly of two large chains, probably two small chains and lipid), a population of approximately 12 SH groups per large chain can be protected from labeling by 5 mM C^{14}-NEM.

Significance to Biomedical Research: Characterization of the ion transport system is essential to an understanding of the regulatory mechanisms of the cardiovascular system.

Taken together the results of several years' work from this laboratory and from other institutions are consistent with, although they do not prove, a model for the ion transport system. This membrane-bound system would consist of an $\alpha_2\beta_2$ complex of two polypeptide subunits with obligatory phospholipids. Although no catalytic role in (Na + K)-ATPase activity can be assigned to the smaller glycoprotein, there is now considerable evidence that either by conformational alteration or changes in association it can regulate the pump activity. The dimer of large chains must traverse the whole membrane with catalytic centers exposed internally and ouabain-binding sites exposed externally. Major conformational changes in this dimer clearly accompany ion transport, but the structural features of the active sites remain obscure.

Further study of the possible role of the glycoprotein in adapting ion fluxes to the needs of the cell should prove fruitful.

Proposed Course of Project: Abandoned on July 15, 1977.

Publication: Takeguchi, C.A., Honegger, U.E., Holland, W.W. and Titus, E.O.: Evidence for subclasses of SH grous in (Na^+ + K^+)-ATPase. LifeSci. 19: 797-806, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HLB 02514-03 PB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Chemical Characterization of Pharmacological Receptors

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

PI:	U. Honegger	Visiting Fellow	PB NHLBI
OTHER:	E. O. Titus	Head, Section on Molecular Pharmacology	PB NHLBI
	W. W. Holland	Chemist	PB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Pulmonary

SECTION
Molecular Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.9	1.3	0.6

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

β-Adrenergic receptors survive in frozen pineals in concentrations sufficient to encourage their characterization from commercial sources. Their analysis is complicated by the presence of substances of unknown significance that cooperatively bind lipophilic adrenergic inhibitors. Binding activity is surprisingly resistant to agents known to inhibit other membrane functions by covalent binding to protein.

1175

Project Description:

Objectives: The molecular mechanisms by which neurohumoral transmitters activate receptors in mammalian smooth muscle, secretory glands, etc., are unknown. The first step is adsorption to a recognition site which exhibits high affinity for a limited range of organic structures related to the natural agonist. Conformational changes associated with this adsorption initiate the response. The goal of this project is to isolate those components of adrenergic and other receptors which serve as agonist-recognition sites in smooth muscle and other autonomically innervated mammalian tissues.

Studies of the nicotinic cholinergic receptor isolated from the electroplax of electric fish together with studies of insulin, angiotensin and other receptors from mammalian tissues indicate that individual receptors are characterized by specific proteins. These may be oligomeric assemblies of subunits, among which are the proteins bearing the recognition sites. The introduction of site-directed, covalently bound radioactive labels into such proteins should be useful in characterizing these sites. Even though the concentrations of adrenergic receptors are minute and the specific agonist-binding properties may not survive isolation, the characterization of a labeled polypeptide, unequivocally identifiable as derived from the receptor, can be of interest, since the information which defines its function is inherent in the amino acid sequence of the protein.

Earlier reports have dealt with the synthesis of potential labeling reagents. This report is concerned with the feasibility of using commercially available bulk tissue, in this case frozen pineal bodies, as sources for receptor studies.

Methods Employed: Concentrations of β-adrenergic receptor are measured by incubation of membrane fractions with ^{3}H-L-dihydroalprenolol (Proc. Natl. Acad. Sci. 72: 1564, 1975), chilling to stabilize the complex with the labeled inhibitor, filtration on glass-fiber filter discs and counting of radioactivity. Specific binding to the adrenergic receptor is characterized by observing the relative abilities of D- and L-propranolol to compete for binding and is routinely measured by observing the displacement of bound radioactivity when 50 μM L-propranolol is included in the incubation mixture.

Major Findings: The concentration of β-adrenergic receptor in insoluble proteins centrifuged at 100,000 x g from homogenates of commercial frozen sheep pineal bodies was 0.50 pmoles/mg of protein, in fair agreement with the average value of 0.65 pmoles/mg from 8 freshly obtained glands. The receptor is reasonably stable on storage, levels of 0.3 to 0.4 pmoles/mg being observed after 5 months. Concentrations in rabbit and beef pineal preparations were 0.2-0.3 and 0.07 pmoles/mg, respectively.

The pineal β-receptors undergo only 28% enhancement of specific activity in a microsomal fraction obtained by differential centrifugation and 59% enhancement in a fraction more dense than the plasma membrane when centrifuged through a discontinuous sucrose gradient. Solubilization of membrane

proteins of 1 M ammonium thiocyante, the most effective of a number of chaotropic salts, gave a 30-50% enhancement of receptor activity in the residual pellet.

Receptor preparations from all three sources, whether or not enriched by the above procedures, contained in addition to the saturable, optically selective receptor a second saturable site to which propranolol and alprenolol bound cooperatively, half saturation occurring at approximately 9×10^{-7} M. The significance of this binding site remains obscure.

Specific binding of ^{3}H-dihydroalprenolol by the β-adrenergic receptor was not influenced by a series of sulfhydryl-seeking, N-substituted maleimides or by reaction with diazotized sulfanilic acid, dicyclohexyl carbodiimide, or periodic acid. Binding was rapidly destroyed by Triton-X-100, concentrations of 0.03% and 0.01% being effective at protein concentrations of 0.3 mg/ml and 2 mg/ml, respectively. Digitonin (1%) removed 76% of the specific binding activity, none of the nonspecific binding and 38% of the total protein. Although receptor activity has been reported in soluble digitonin extracts of muscarinic receptors and turkey erythrocytes, no specific binding could be demonstrated in digitonin extracts of pineal membranes by a gel filtration procedure using Sephadex G-50.

Significance to Biomedical Research: Identification of those molecules which bind hormones and transmitters at the receptor sites could contribute to an understanding of physiological regulatory mechanisms. Autoimmune responses to receptor proteins have been implicated in myasthenia gravis and may play a role in other diseases. The molecular basis for the selective action of β-2 agonists on bronchial receptors and for the age-dependent loss of β-response in rat vascular tissue is unknown.

Proposed Course of Action: Project abandoned July 1, 1977.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HLB 02518-02 PB

PERIOD COVERED: July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Factors Affecting the Binding of Drugs to Plasma Albumin

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

PI:	C. F. Chignell	Pharmacologist	PB NHLBI
	S. W. Boobis	Guest Worker	PB NHLBI
	D. B. Millar		

COOPERATING UNITS (if any)

S. W. Boobis is supported by a fellowship from Hoffmann-La Roche Inc., Nutley, N. J. 07110. D. B. Millar is Chief of the Laboratory of Physical Biochemistry, NMRI, Bethesda, Maryland 20014

LAB/BRANCH

Pulmonary

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NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.45	0.35	0.1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The effect of various factors, including disease and protein concentration, on the binding of drugs to plasma albumin has been investigated. The binding of several acidic drugs to albumin from uremic and neonatal subjects was reduced. While there were differences in the amino acid composition of albumin from uremics and neonates, no differences in their immunological properties were observed. Isoelectric focussing separates normal albumin into two components with isoelectric points of 4.8 and 5.0. Both the uremic and the neonatal albumins had differing amounts of these two components when compared to normal albumin. The reduction in drug binding observed when albumin concentration is increased is not due to polymerization of the protein but may result from a change in protein conformation.

1178

PHS-6040
(Rev. 10-76)

Objectives: The binding of a drug to plasma albumin can dramatically alter its rate of distribution, metabolism and excretion. Drug binding to plasma albumin is reduced in certain disease states, e.g. uremia (Ann.N.Y.Acad.Sci. 226: 115, 1973; Ann.N.Y.Acad.Sci. 226: 101, 1973). The amino acid composition and electrophoretic properties of albumin isolated from uremic patients and neonates has therefore been studied. It has also been reported that the affinity of certain drugs for albumin decreases as the concentration of albumin increases (J.Pharm.Sci. 63: 1698, 1975). This phenomenon has been investigated by a variety of physical techniques in an attempt to provide a molecular basis for the observation.

Methods: Plasma samples were obtained from healthy volunteers, patients with renal and hepatic disease, and from newborn infants. Albumin was isolated using the affinity chromatographic procedure of Travis and Pannell (Clin.Chim. Acta 49: 49, 1973).

Major Findings: Sulfadiazine binding was measured in plasma from normal and uremic subjects. A reduction in both the number of binding sites and in the affinity of sulfadiazine for the sites was observed. Amino acid analysis of isolated plasma albumin indicated significant differences in the threonine, serine, proline, alanine, methionine and tyrosine content of the uremic albumin. Analytical scale isoelectric focussing of the purified albumin revealed the presence of two protein bands with isoelectric points of 4.8 and 5.0. Marked differences in the relative amounts of protein present in the two bands were observed when albumins from normal and uremic subjects were compared. However, immunodiffusion studies showed that both albumins reacted with rabbit antiserum to human albumin to give a single preciitin line with no spurring. These observations suggest that the difference in binding between normal and uremic patients may result from quantitative differences between the two forms of albumin that are separated by isoelectric focussing.

Plasma albumin isolated from neonates had a reduced threonine, valine and methionine content but an increased content of serine, glycine and lysine. Neonatal albumin showed only one band by isoelectric focussing with an isoelectric point of 4.8. Immunodiffusion studies with neonatal and adult albumin produced a single precipitin line with no spurring. These observations suggest that the albumin of neonatal plasma is different to that of adult in that it contains only one component. This may account for the different binding properties of the two albumins.

The molecular properties of plasma albumin were studied as a function of concentration in an attempt to understand why drug binding is dependent upon the concentration of albumin. Sedimentation equilibrium measurements indicated that there was no concentration-dependent aggregation of albumin. No change in the hydrodynamic properties of albumin were detected over a wide range of protein concentrations. However, as the concentration of albumin is increased from 0.1% to 4%, a decrease in the α-helical and β-pleated sheet content was observed with a concomitant increase in the amount of random coil. This change was not observed in albumin that had been intramolecularly

cross-linked with dimethylsuberimidate. These results suggest that reduced drug binding observed at high albumin concentrations may be due to a change in the conformation of the protein.

Significance: Since the binding of a drug to plasma albumin can alter the rate of distribution, metabolism and excretion, it is important to understand all of the factors that can affect binding.

Proposed Course: This project is now complete. No further work is planned.

Publications:

Wallace, S. (Boobis, S.W.): Altered plasma albumin in relation to the newborn infant. Br.J.Clin.Pharmac. 4: 82-85, 1977.

Boobis, S.W.: The alteration of plasma albumin in relation to decreased drug binding in uremia. J.Lab.Clin.Med., in press.

Chignell, C.F.: Ligand Binding to Plasma Albumin. In Fasman, G.D. (Ed.): CRC Handbook of Biochemistry and Molecular Biology (ed. 3). Cleveland, CRC Press, 1976, vol. 2, pp. 554-582.

Chignell, C.F.: Protein Binding. In Hirtz, J.L. and Garrett, E.R. (Eds.): Drug Fate and Metabolism: Methods and Techniques. New York, Marcel Dekker Inc., 1977, vol. 2, pp. 87-228.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HLB 02520-02 PB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Inhibition of Cell Growth and Proliferation in Culture by Anti-Inflammatory Drugs

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

PI:	M. A. Beaven	Pharmacologist	PB NHLBI
	M. C. F. de Mello	Visiting Fellow	PB NHLBI
OTHER:	H. S. Kruth	Staff Associate	PB NHLBI

COOPERATING UNITS (if any)
Dr. H. S. Kruth, Laboratory of Cellular Metabolis, National Heart, Lung, and Blood Institute, Bethesda, Maryland 20014

LAB/BRANCH
Pulmonary

SECTION
Molecular Pharmacology

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NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

As observed previously with hepatoma cell cultures, nonsteroidal anti-inflammatory drugs inhibited proliferation of human fibroblast cells in culture. The order of potency of these drugs in inhibiting growth was similar to that reported for their anti-inflammatory activity. The drugs did not alter cell viability, and their action was reversible. After a short delay (18 hr), exponential growth resumed once the drug was removed from the culture. These kinetics suggest the possibility that the cells had been arrested in the G_1 phase of growth. No evidence was found that the anti-inflammatory drugs acted through inhibition of prostaglandin synthesis. Exogenous prostaglandins did not reverse the inhibition, and prostaglandin synthesis could not be detected in the cultures.

1181

Introduction: Our earlier studies have shown that indomethacin and aspirin in pharmacological doses inhibit growth and metabolic activity of a transplantable mast cell ascites tumor (P815) and Lewis lung carcinoma in mouse (Hial et al., Eur.J.Pharmac. 37: 367-376, 1976). More detailed studies with cell cultures showed that all the common nonsteroid anti-inflammatory drugs inhibit cell proliferation when added to rat hepatoma cultures. A parallel reduction in protein and nucleic acid synthesis, as measured by isotope incorporation, was also observed. Since the effect on protein and nucleic acid synthesis was apparent only after cell replication was retarded, it was felt that the primary action of the drugs was on cell proliferation. Pharmacologically inactive derivatives, such as m-hydroxybenzoic acid and gentisic acid, were not inhibitory in concentrations up to 5 mM. The order of potency in inhibiting culture growth, meclofenamate > indomethacin > salicylamide > phenylbutazone > phenacetin > aspirin = salicylic acid, was similar to that reported for their anti-inflammatory activity and their ability to inhibit prostaglandin synthesis.

The present work is a continuation of these studies. Experiments were undertaken to determine if

1) the anti-inflammatory drugs inhibited growth of a nontransformed cell line, the fibroblast,

2) the effects of the anti-inflammatory drugs were reversible,

3) the drugs acted through alteration of prostaglandin synthesis.

Methods Employed: Hepatoma cell cultures were obtained from a line started by E. G. Thompson (Biochem.Biophys.Res.Commun. 56: 605, 1974). Human fibroblasts were grown from the foreskin of 1-day-old males and were supplied to us by Dr. Vincent Manganiello. The cells were grown in Eagle's medium #2 with 10% fetal calf serum under 95% air and 5% CO_2 at 37°C. Cultures were grown in large disposable screw cap flasks. Fresh cultures were prepared each week. For individual experiments suspensions of 4-day-old cultures were diluted with 20 volumes of fresh medium. One milliliter aliquots of the diluted culture (approximately 50 x 10^3 cells) were dispensed into 1 ml wells of Costar cluster plates. Drugs were added to cultures either at the start of culture growth (day 0) or day 2 of culture growth. To remove drug the cultures were washed once with Eagle's medium and fresh medium was then added. For determination of cell number, the cells were treated with trypsin and counted by use of a Neubauer counting chamber.

Measurement of ^{3}H-leucine incorporation. The labeled amino acid was added to each well, and the culture plates were incubated for 3 hr at 37°, after which the medium was removed. The individual culture wells were rinsed carefully 2 times with 0.5 Locke's solution, once with 0.5 ml ice-cold 10% trichloroacetic acid (TCA) solution, and finally with 1.0 ml of ethanol. The TCA precipitate was dissolved in 0.2 ml Hyamine hydroxide, transferred to a glass scintillation vial and dissolved in a liquid scintillation cocktail.

Studies with arachidonic acid. Arachidonic acid-[1-^{14}C] (50 mCi/20 µl) was added to each well on day 3 of growth and the cultures were incubated for a further 20 hr. Aliquots of the medium were removed for assay of radioactivity, and the remainder of the medium was removed by aspiration. The cultures were washed and treated with TCA, as described above. The TCA precipitate was washed twice with water (0.5 ml) and extracted with 1.0 ml ethanol. Aliquots, ~50 µl, of the ethanol extract were assayed for radioactivity. The remainder was evaporated to dryness under a stream of N_2 and redissolved in an ethanolic solution of the various unlabeled prostaglandins and arachidonic acid (50 µg of each). This mixture was chromatographed on TLC silica gel plates in a solvent system consisting of benzene:p-dioxane:glacial acetic acid (200:200:10 parts by volume) (Green and Samuelsson, J.LipidRes. 5: 117, 1964). Segments were scraped from the plates and assayed for radioactivity.

Identification of the arachidonic acid metabolite. One major metabolite of ^{14}C-arachidonic acid was observed on the chromatogram. This compound was not identified but was assumed to be a "phospholipid" on the following evidence. The labeled metabolite was present exclusively in the particulate fraction of the cells and was precipitated by 0.4 perchloric acid. It was readily extracted from the perchloric acid precipitate into ethanol. When chromatographed in the solvent system described above, the metabolite remained at the origin. Mild alkaline hydrolysis (0.5 M N-OH, in 75% methanol at -37° for 45 min) according to the procedure of Lands et al. (Biochim.Biophys.Acta 164: 426, 1968) resulted in the disappearance of the labeled metabolite and reappearance of a material which migrated as arachidonic acid.

Results. 1) Inhibition of fibroblast proliferation. Growth of human fibroblast cultures was inhibited by all drugs tested. These included indomethacin, phenylbutazone and sodium salicylate. ^{3}H-Leucine incorporation and cell numbers were reduced proportionately. As in the hepatoma cell cultures, the inhibition was progressively greater with higher concentrations of drugs. The ID_{50} (concentration required for 50% inhibition of growth) values were similar to those observed with the hepatoma cell cultures.

2) Reversibility of the effect of the anti-inflammatory drugs. Although cell counts were reduced in cultures grown in the presence of the anti-inflammatory drugs, the proportion of viable cells (as measured by trypan blue exclusion) was not reduced. In studies with 0.4 mM indomethacin (a concentration that is sufficient to stop growth completely), the proportion of cells that were viable ranged from 90-96% over the course of 8 days. In control cultures, the number of viable cells declined (to around 80% of total) as the cultures became confluent (day 5), and cells began to slough from the bottom of the wells (days 7 and 8).

In cultures exposed to 0.4 mM indomethacin, normal growth resumed and drug was removed. There was a short delay, 16 hr, before cells began to proliferate. Thereafter, cell numbers increased exponentially with the same doubling time as for cultures grown without drug. The short delay and immediate resumption of logarithmic phase of growth could indicate that the cells had become synchronized.

Other studies showed that the effects of aspirin (see last year's report) and phenylbutazone (Table 1) were also reversible.

3) Inability of prostaglandin to reverse the effects of anti-inflammatory drugs. The inhibition of growth of hepatoma cell cultures by indomethacin was not reversed but was enhanced by the addition of the prostaglandins (PG). The additional inhibition appeared to be due to that produced by the PG's alone. All the PG's tested inhibited culture growth to a greater ($PGA_{1\&2}$ and $PGE_{1\&2}$) or lesser ($PGB_{1\&2}$ and $PGF_{2\alpha}$) extent. The inhibition, however, differed from that induced by indomethacin in that it was not reversible (Table 1).

TABLE 1

Inhibition of ^{3}H-leucine incorporation by indomethacin and prostaglandins: reversibility

Addition	^{3}H-Leucine incorporated	
	Unwashed	Washed
	cpm *(% of control)*	
Control (no drug)	82,832 ± 1,761 *(100)*	112,860 ± 3,024 *(100)*
Phenylbutazone, 0.5 mM	402 ± 33 *(0.5)*	93,574 ± 4,899 *(83)*
Indomethacin, 0.2 mM	8,958 ± 219 *(11)*	71,966 ± 4,725 *(64)*
PGA_1, 10 µg/ml	89 ± 5 *(0.1)*	441 ± 68 *(0.4)*
PGE_2, 10 µg/ml	11,281 ± 4,422 *(14)*	23,741 ± 6,192 *(21)*

Values are mean ± SEM (n = 6). Drugs were added at the start of culture growth. On the 3rd day of growth, the cultures were washed two times with fresh medium or left unwashed. The cultures were reincubated for an additional 4 days before measurement of ^{3}H-leucine incorporation.

4) Effect of the anti-inflammatory drugs on prostaglandin synthesis and arachidonic acid metabolism. Prostaglandin synthesis was not observed in the hepatoma cell cultures. Prostaglandin levels as measured by radioimmunoassay did not increase in the culture medium during growth (Dr. Zussman, personal communicaton), and the formation of prostaglandins was not apparent when the cultures were incubated with ^{14}C-arachidonic acid and the labeled metabolites separated by chromatography. Most of the ^{14}C-arachidonic acid was slowly incorporated (62-70% by 24 hr and 96% by 48 hr) into the "phospholipid" fraction (see "Methods"). Although this incorporation was inhibited by indomethacin, other anti-inflammatory drugs did not share this effect.

Significance to biomedical research and proposed course of project. The studies indicate that all the common anti-inflammatory drugs reversibly inhibit growth and metabolic activity of hepatoma cell cultures. Since their order

of potency appears to parallel their efficacy as anti-inflammatory agents, the findings may have some bearing on the anti-inflammatory properties of these drugs. Since no evidence could be obtained to indicate that the effect of the drugs was due to interference of prostaglandin synthesis, alternative mechanisms for the inhibition of cell proliferation will be investigated. The possibility that cells become synchronous in the presence of these drugs will also be explored.

Publications:

Hial, V., de Mello, M.C.F., Horakova, Z. and Beaven, M.A.: Effect of anti-inflammatory drugs on growth and metabolic activity of mammalian cell cultures. J.Pharmac.Exp.Ther., in press.

Hial, V., Horakova, Z., Shaff, R.E. and Beaven, M.A.: Alteration of tumor growth by aspirin and indomethacin: Studies with two transplantable tumors in mouse. Eur.J.Pharmac. 37: 367-376, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HLB 02523-01 PB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Molecular Mechanisms of Mast Cell Degranulation

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

PI:	M. J. Ortner	Guest Worker	PB NHLBI
	C. F. Chignell	Pharmacologist	PB NHLBI

COOPERATING UNITS (if any)

Dr. Ortner holds a National Research Service Postdoctoral Fellowship from the Division of Research Grants, NIH

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.9	0.1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

We have used fluorescent and spin-labeled probes to investigate the binding of compound 48/80 to cell membranes. The fluorescent probe diphenylhexatriene has shown that mastocytoma cells, like some other forms of neoplastic cells are more fluid in the hydrophobic region of the cell membrane than their normal counterparts. Fluorescent-labeled 48/80 binds to mastocytoma cells exhibiting a fluorescence polarization value which is similar to the calcium ionophores X-537A and A-23187. Fluorescence quenching measurements indicate that the 48/80 binding site is close to that of stearic acid; however, unlike the fatty acid, the mobility of bound 48/80 is unaffected by detergents. Spin-labeled fatty acid probes have shown that the hydrophilic regions of mastocytoma and mast cell membranes are similar in fluidity. Inactive spin-labeled 48/80 polymers do not bind to mastocytoma cell membranes; however, the active polymers bind strongly to multiple sites which are inaccessible to the extracellular aqueous environment. Polymyxin B displaced 48/80 from these sites; however, morphine, curare and concanavalin A had no effect. Fluorescent and spin-labeled probes have been successfully adapted to the study of the action of compound 48/80 on the molecular level.

1186

Objectives: Mast cell degranulation releases histamine and other pharmacological mediators which results in the symptoms of many respiratory disorders (i.e. asthma, hay fever). In order to understand better the underlying mechanisms behind this form of noncytotoxic secretion, we have studied the artificial histamine liberator, compound 48/80. This drug has been successfully labeled with both fluorescent and spin-labeled moieties. In addition, other spectroscopic probes have been used to study mast cell and mastocytoma cell membranes. With the aid of these tools, we are trying to identify the binding site(s) of 48/80 in order to elucidate the molecular mechanism of histamine secretion from mast cells.

Methods: Rat mast cells were isolated and purified according to well-established procedures (Perera, B.V.A. and Mongar, J.L.: Immunology 6: 472, 1963). The mastocytoma cells were an established cell line (P-815 ascites mastocytoma) grown in CDF-1 mice. The spin label probes were studied with a Varian E-4 ESR spectrometer while the fluorescent probes were studied with an Aminco-Bowman spectrophotofluorometer.

Findings: 1) Spin-Labeled Probes--All of the cell types mentioned above intercalate spin-labeled 5-doxyl-stearic acid (SL-stearic acid) into their plasma membranes. Fluidity measurements using SL-stearic acid suggest that there is no significant difference between the membranes of mast cells and other cell types. No distinct phase transitions were observed in the membranes of either the mastocytoma cells or the mast cells over the range 0-40^{o}. While compound 48/80 does not affect membrane fluidity, it does bind to membrane-intercalated SL-stearic acid, causing a decrease in the ESR signal.

Spin-labeled 48/80 (SL-48/80) has been synthesized and purified. The active fractions (i.e. those which liberate histamine from mast cells) bind to mastocytoma cell membranes in a manner which suggests multiple binding sites. The inactive polymers do not bind to the cells. Unlike SL-stearic acid, membrane-bound SL-48/80 was unaffected by nonionic detergents. While cell-bound SL-48/80 was not exposed on the surface of the cells, it was displaced by unlabeled 48/80 and polymyxin B. The binding of 48/80 was unaffected by concanavalin A, curare, or morphine. Human erythrocyte ghosts and liposomes bound SL-stearic acid and SL-48/80 in a manner similar to intact cells, and the effects of detergents were also similar.

2) Fluorescent Probes--Diphenylhexatriene (DPH) was intercalated into the hydrophobic region of mast cells and mastocytoma cells. Fluorescence polarization measurements over a range of 0-40^{o}C showed a gradual increase in membrane fluidity; however, no abrupt phase transitions occurred. In contrast to the region probed by SL-stearic acid, the hydrophobic region of the mast cells was significantly less fluid than that of the mastocytoma cells.

Calcium ionophores X-537A and A-23187, which cause histamine release from mast cells, were also studied as fluorescent probes. Their mobility in mastocytoma membranes was unaffected by temperature changes between 0-40^{o}; however, calcium increased the polarization 30-50%, indicating the formation of a calcium-ionophore complex within the membrane. Compound 48/80 had no

effect on the fluorescence quantum yield and polarization of either DPH or the ionophores.

Two fluorescent fatty acids probes [2-(9-anthroyl)-palmitic acid and 12-(9-anthroyl)-stearic acid] were synthesized. In mastocytoma cells, spin-labeled 48/80 quenched the fluorescence intensity of the anthroyl moieties, regardless of their position on the fatty acid chain. This indicated that 48/80 came into very close contact with the fatty acid in all regions of its membrane penetration. Anthroyl-48/80 has also been synthesized and studied. The fluorescence polarization of anthroyl-48/80 bound to mastocytoma cells is similar to that of the ionophores. Also, like the ionophores, its degree of polarization was apparently unaffected by temperature.

Conclusion and Proposed Course of the Project: The above indicates progress made in adapting both fluorescent and spin-labeled probes to the study of mast cells and compound 48/80. With the aid of these techniques, we have demonstrated many heretofore unknown properties of mast cells, mastocytoma cells and 48/80. These studies will be amplified further with the goal of identifying the binding site(s) of compound 48/80 and its mechanism of action on the molecular level. The strong binding of 48/80 to stearic acid indicates that it may act by interfering with lipid organization within the membrane. This will be further investigated. In addition, we wish to study the effects of various agents which interfere with the action of 48/80 and to clarify further certain molecular aspects of the secretion phenomenon.

Publications: Sinha, B.K., Cysyk, R.L., Millar, D.B. and Chignell, C.F.: Synthesis and biological properties of some spin-labeled 9-aminoacridines. J.Med.Chem. 19: 994-998, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HLB 02524-01 PB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Mechanism of Drug-Induced Photosensitivity

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

PI: C. F. Chignell Pharmacologist PB NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Pulmonary

SECTION
Molecular Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The role of singlet oxygen in drug-induced photosensitization has been investigated by means of electron spin resonance. Irradiation of anthracene, 9-aminoacridine, indoxole, chlortetracycline and declomycin with visible light produced high concentrations of singlet oxygen. By contrast, the sulfonamide drugs chlorthiazide, tolbutamide and sulfadiazine did not generate singlet oxygen on light irradiation. These studies indicate that singlet oxygen may be involved in the photosensitization caused by certain drugs.

1189

Objectives: Many drugs are known to cause photosensitivity in certain individuals. The photosensitive response may be one of two types, either phototoxic or photoallergic. The phototoxic reaction generally occurs during a patient's first exposure to sunlight after taking the drug and usually takes the form of an exaggerated erythemal response. Photoallergic individuals may also exhibit an initial erythemal reaction. As this subsides, delayed abnormal responses begin to appear including papular, eczematous and urticarial reactions. Such reactions to light may persist for months after avoidance of the photosensitizer.

While the initial step in all forms of photosensitivity must be the absorption of light by the drug or its metaboliters, the precise mechanism of phototoxicity and photoallergy is still unknown. In previous studies we have shown that free radicals may be involved in the photosensitizing reaction. In this report we examine the possible role of singlet oxygen.

Methods Employed: Lion and co-workers (Nature 263: 442, 1976) have shown that singlet oxygen can be detected by electron spin resonance (ESR) spectroscopy. Ethanol solutions (4 ml) of the drug (1 mM) and 2,2,6,6-tetramethylpiperidine (0.1 M), I, contained in a 1 cm^2 cuvette were irradiated through window glass by a 150 W Xenon arc lamp at a distance of 12 in. Samples were withdrawn in glass capillaries at fixed time intervals, and the concentration of 2,2,6,6-tetramethylpiperidine-1-oxyl (II), generated by singlet oxygen oxidation of I, was determined with the aid of an ESR spectrometer.

Major Findings: High concentrations of singlet oxygen were produced during the irradiation of anthracene and 9-aminoacridine (Table 1). This observation is consistent with the known photodynamic properties of these compounds. Among the drugs which were examined, indoxyl produced the highest concentration of singlet oxygen. Indoxole-induced singlet oxygen generation was enhanced in deuterated ethanol and was abolished by the addition of sodium azide (Table 2), a known quencher of singlet oxygen.

Among the tetracyclines chlortetracycline and declomycin produced the highest levels of singlet oxygen while ninocycline and doxycycline were found to be inactive. These observations are in accord with the recent studies of Wiebe and Moore (J.Pharm.Sci. 66: 186, 1977), who have shown that irradiation of aerated aqueous solutions of tetracycline gives rise to oxygen uptake.

None of the sulfonamide drugs were found to generate singlet oxygen during irradiation, suggesting that the photosensitization caused by these compounds may be mediated by a different mechanism.

Significance: Photosensitization is an undesirable side effect which is shared by many drugs with widely different structures. A better understanding of this phenomenon should lead to the design of drugs which are not photosensitizers.

Proposed Course: The role of singlet oxygen in the photosensitivity produced by other drugs will be investigated.

1190

Publications: None

TABLE 1

The Generation of Singlet Oxygen During the Irradiation of Various Drugs

Drug	Concentration of II after irradiation for 21 minutes ($M \times 10^{5}$)
Anthracene	67.0
9-Aminoacridine	48.0
Indoxole	18.8
Chlortetracycline	12.1
Declomycin	12.0
Trichlorosalicylanilide	9.6
Oxytetracycline	6.0
Tribromosalicylanilide	5.0
Tetracycline	2.1
Griseofulvin	1.2
8-Methoxypsoralen	1.0
p-Aminobenzoic acid	1.0
Hexachlorophene	0.4
Minocycline	0.2
Doxycycline	--
Bithional	--
Chlorthiazide	--
Tolbutamide	--
Sulfadiazine	--

TABLE 2

The Generation of Singlet Oxygen During the Irradiation of Indoxole

Solvent	Concentration of II after irradiation ($M \times 10^{5}$)
Ethanol	18.8
d_6-Ethanol	38.7
Ethanol + 10^{-2} NaN_3	0.8
Ethanol saturated with O_2	23.1

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PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Spin Label Studies of Cholinergic Enriched Membranes from Torpedo californica

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

PI: C. F. Chignell Pharmacologist PB NHLBI
P. W. Taylor

COOPERATING UNITS (if any)
Dr. Palmer W. Taylor, Division of Pharmacology, Department of Medicine, School of Medicine, University of California, San Diego, La Jolla, Calif., 92037

LAB/BRANCH
Pulmonary

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Molecular Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The binding of a nitrogen substituted mononitroxide congener (I) of decamethonium to membrane-bound cholinergic receptor from Torpedo californica has been monitored by means of electron spin resonance. The receptor exhibited a twofold increase in affinity for I over a period of 30 min after initial mixing. One mole of spin label I is bound per mole of cobra α-toxin binding site and the label is completely dissociated from the complex by the addition of excess toxin.

1192

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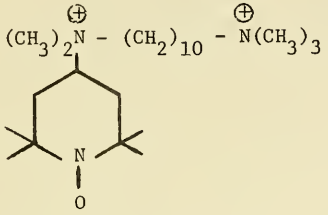
(Rev. 10-76)

Objectives: Recent studies by Weber and co-workers (Proc.Natl.Acad.Sci. 72: 3443, 1975) have shown that the apparent competition between cholinergic agonists and cobra α-toxin for binding sites on the cholinergic receptor is dependent on the duration of agonist exposure. One of the possible explanations for this phenomenon is a time-dependent increase in the affinity of the receptor for the ligand. The object of this study was to demonstrate this increase directly with the aid of a spin-labeled analog (I) of decamethonium.



I

Methods: The synthesis of I has been previously described (J.Med.Chem. 18: 669, 1975). The cholinergic receptor-enriched membranes were prepared by the procedure of Flanagan and co-workers (J.Biol.Chem. 251: 858, 1976).

Major Findings: In the presence of the receptor-enriched membranes, the nitroxide group of I became highly immobilized. When binding was measured as a function of time, a gradual decrease in the concentration of free spin label was observed over a period of about 30 min. This decrease represented a 2- to 3-fold increase in the affinity of I for the receptor site. Scatchard plots indicated that one molecule of I was bound per toxin site with a dissociation constant of 1.7×10^{-6} M at 30 min. The addition of α-toxin caused complete dissociation of the receptor complex with spin label I.

Significance: Since many drugs exert their pharmacological effect by binding to the cholinergic receptor, it is important to understand the mechanism of this interaction.

Proposed Course: This project is now complete.

Publications:

Wee, V.T., Sinha, B.K., Taylor, P.W. and Chignell, C.F.: Interation of spin-labeled bisquaternary ammonium ligands with acetylcholinesterase. Mol.Pharmac. 12: 667-677, 1976.

Weiland, G., Georgia, B., Lappi, S., Chignell, C.F. and Taylor, P.: Kinetics of agonist mediated transitions in state of the cholinergic receptor. J.Biol.Chem., in press.

1193

Weiland, G., Georgia, B., Wee, V.T., Chignell, C.F. and Taylor, P.: Ligand interactions with cholinergic receptor enriched membranes from Torpedo: Influence of agonist exposure on receptor properties. Mol.Pharmac. 12: 1091-1105, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HLB 02526-01 PB

PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

A Magnetic Resonance Study of the Structure of Compound 48/80

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

PI:	C. F. Chignell	Pharmacologist	PB NHLBI
	M. J. Ortner	Guest Worker	PB NHLBI
	E. A. Sokoloski	Chemist	LC NHLBI

COOPERATING UNITS (if any)

Dr. Ortner holds a National Research Service Individual Postdoctoral Award from the Division of Research Grants, NIH. Mr. Sokoloski is a chemist in the Laboratory of Chemistry, NHLBI

LAB/BRANCH

Pulmonary

SECTION

Molecular Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

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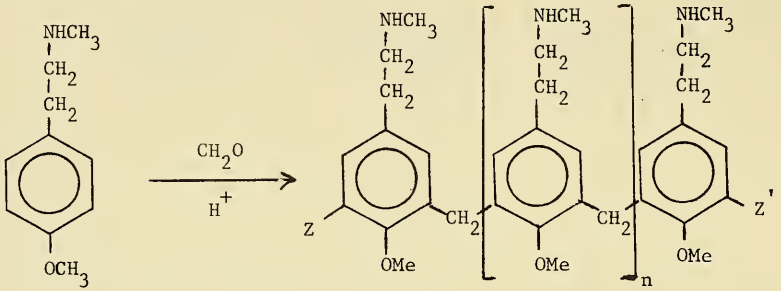
☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The structure of compound 48/80, a potent liberator of histamine from mast cells, has been studied by Carbon-13 nuclear magnetic resonance (NMR). Compound 48/80 is a polymer prepared by heating 4-methoxy-N-methylphenethylamine with formaldehyde in acid solution. The NMR spectrum indicates that 48/80 is a polymer in which bridging methylene groups link the 4-methoxy-N-methylphenethylamine in the 3 and 3' positions. The polymer chains are terminated by CH_2OH groups. Light scattering measurements indicate that at concentrations above 2 mg/ml compound 48/80 forms micelles. This is reflected in the NMR spectrum by the sharpness of the N-CH_3 and the α-carbonatom of the phenethylamine chain.

1195

Objectives: Compound 48/80 was first synthesized by Baltzly and co-workers (J.Amer.Chem.Soc. 71: 1301, 1949) by condensing 4-methoxy-N-methylphenylethylamine (I) with formaldehyde under acidic conditions. The resultant mixture of polymers has been shown to be a potent liberator of histamine from mast cells (Br.J.Pharmac. 6: 499, 1951). While the pharmacological properties of 48/80 are well-known, the chemical structure has not been completely elucidated. The reaction is usually represented as



(I) (II)

where Z and Z' may be H, CH_2OH, CH_2Cl. Gel filtration studies by Read and Lenney (J.Med.Chem. 15: 320, 1972) indicate that the active fractions range in free base molecular weight from 700 to 1400. These molecular weights suggest that the degree of polymerization ranges from tetramer (n = 2) to octamer (n = 6) with the average being the hexamer (n = 4).

In this study, we have attempted to use carbon-13 nuclear magnetic resonance (NMR) to verify the proposed structure for 48/80.

Methods: NMR spectra were recorded on a JEOL FX-60 spectrometer operating at 15.03 MHz for carbon-13. Samples were dissolved in D_2O to give a final concentration of 0.1 M-0.2 M with respect to the monomer. Dioxane was used as an internal standard.

Major Findings: The 3 and 3' carbon atoms of I are absent in 48/80 (Table 1), thus confirming that polymerization occurs at these carbon atoms. When carbon-13-enriched formaldehyde was employed, two new peaks were visible at 30.62 ppm (T) and 59. 52 ppm (T). While the new peak at 30.62 ppm can be attributed to the bridging methylene groups in 48/80, this resonance is at higher field than the corresponding carbon atom of diphenylmethane (δ_c = 37.0 ppm). One possible explanation for this observation may be that steric crowding forces the phenyl rings of the polymer to twist out of coplanarity so that the deshielding effect of the π-orbital system is minimized. The resonance at 59.52 ppm is much closer to the methylene resonance of benzyl alcohol (δ_c = 64.7) than benzyl chloride (δ_c = 46.6), suggesting that in 48/80 the terminating groups (Z and Z' in II) are CH_2OH.

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It is of interest that carbon atom 4 in I (δ_C = 158.86) is replaced by two resonances (δ_C = 155.68 and 157.18) in the 48/80 polymer. These resonances could be due either to the same carbon atom in two different environments or to the presence of two different carbon atoms. Attempts to cause coalescence of these two resonances by heating or changing solvent were unsuccessful, suggesting they result from two different carbon atoms. These resonances may represent carbon atoms at the end of the polymer and in the middle of the polymer. It should also be pointed out that the OCH_3 resonance of I is also replaced by two resonances in the 48/80 polymer.

It was also noted that resonances due to the $N\text{-}CH_3$ and the α-carbon atom of the phenethylamine chain were much sharper than the remaining resonances. Light scattering experiments indicated that above a concentration of 0.01 M 48/80 forms micelles. Thus the sharpness of the $N\text{-}CH_3$ and the α-carbon atoms is probably due to the greater mobility of the phenethylamine side chain.

Significance: Mast cell degranulation is probably one of the major causes of lung disease. Furthermore, anaphylactic shock is due to a massive release of histamine from mast cells. Compound 48/80 is a model compound which is representative of a large and diverse group of chemicals, e.g. polymyxin B, concanavalin A and dextran. Elucidation of the mechanism 48/80-induced degranulation may shed light, not only on the means by which various chemical agents induce histamine release, but may also provide a clue as to the mechanism of IgE-induced mast cell degranulation.

Proposed Course: The mechanism of mast cell degranulation will be studied with the aid of various spectroscopic probes including fluorescence and electron spin resonance.

Publications: None

TABLE 1

Chemical Shifts[a] of the Carbon Atoms in p-Methoxy-N-methylphenethylamine (I) and 48/80 (II)

Carbon[b]	Structure I[c] δ_c (ppm)	Structure II[c] δ_c (ppm)
β	31.58 (T)	31.77 (T)
N-CH_3	33.59 (Q)	33.59 (Q)
α	51.00 (T)	50.74 (T)
OCH_3	56.27 (Q)	56.59 (Q), 61.50 (Q)
3,3'	115.39 (D)	--
1	129.69 (S)	--[d]
2,2'	130.69 (S)	--[d]
4	158.86 (S)	155.68 (S), 157.18 (S)
$^{*}CH_2OH$	--	59.52 (T)
$-^{*}CH_2-$	--	30.62 (T)

[a] Shift obtained with p-dioxane as an internal standard and converted to TMS using 67.4 ppm as shift for dioxane.

[b] Carbon-13-enriched carbons are marked with an asterisk.

[c] Off resonance identified the peaks as singlet (S), doublets (D), or triplets (T).

[d] These peaks could not be resolved.

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PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Spin Label Studies of Horseradish Peroxidase

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

PI:	G. Rakshit	Visiting Fellow	PB NHLBI
	C. F. Chignell	Pharmacologist	PB NHLBI

COOPERATING UNITS (if any)

None

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Molecular Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20014

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

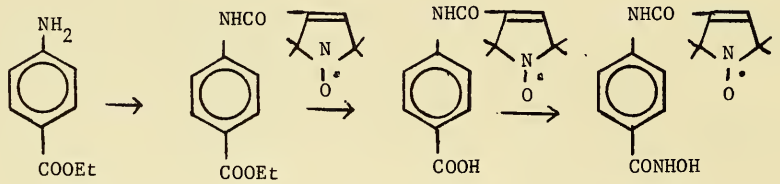
The topography of the active site of Horseradish Peroxidase (HRP) and manganic HRP has been studied with the aid of a spin-labeled analog of benzhydroxamic acid. The electron spin resonance measurements indicated that the nitroxide moiety of this spin label when bound to HRP became highly immobilized. The $2T_{\perp}$ varied inversely with temperature reaching a maximum of 68.25 G at $0^{\circ}C$ and a minimum of 46.5 G at $52^{\circ}C$. From the dipolar interaction between iron atom and free radical, the minimum distance between the two spins was estimated to be 14 Å.

1199

Horseradish peroxidase (HRP) is a hemeprotein enzyme that primarily catalyzes the oxidation of electron donor molecules by peroxides. As in met Hb/Mb, the sixth ligand position of its iron atom is presumably occupied by H_2O and can be replaced to accommodate various exogenous ligands including O_2 and H_2O_2. The object of the present study is to examine the active site topography of HRP with the aid of a spin label.

HRP was cleaved into the heme and apoenzyme by a modification of Teale's method (T. Yonetani, J.Biol.Chem. 242: 5008, 1967). Mn(III) and Zn(II) HRP were prepared by reacting concentrated solution of apoHRP with corresponding metalloporphyrins. Manganic HRP was purified through DEAE cellulose column, while Zinc HRP was passed through a sephadex G-25 column to remove uncomplexed porphyrin.

The benzhydroxamic acid spin label (I) was synthesized from benzocaine, using the following reaction sequence,



(II) (I)

Spin label I forms a stable and noncovalent complex with HRP with a 4 nm hyperchromic shift in the Soret band of the enzyme at 407 nm. In the visible absorption region, the complex shows bands at 504 nm and 638 nm compared to those of the native enzyme at 497 nm and 641 nm. This distinct absorption spectrum remains unaltered between pH 4 and 8.

The ESR spectrum of the bound spin label I exhibits a wide separation (59.2 G) between the extreme low field and high field peaks and is characteristic of a strongly immobilized nitroxide group. From the linear Scatchard plot obtained from titration of HRP by spin label I, it was estimated that HRP has a single binding site with an association constant $K_a = 2 \times 10^5\ M^{-1}$.

The affinity of HRP for an analogous spin label II was too weak to be detected from our ESR spectra. The spin label I seemed to be very specific for HRP and did not bind any other hemeproteins, e.g. Mb, cytochrome c, catalase, or lactoperoxidase, examined so far.

The binding of spin label I with HRP was completely inhibited on addition of H_2O_2, CN^-, F^-, or benzhydroxamic acid, all of which formed spectroscopically distinct complexes with HRP. This would confirm that the spin label II indeed competes for the same binding domain on HRP. Addition of paramagnetic

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metal ions, e.g. $CuCl_2$, $K_4Fe(CN)_6$, or $NiCl_2$, also abolished spin label I binding to HRP, presumably due to the formation of metal salts with hydroxamic acid group of spin label I. Treatment of HRP with 6 M guanidine HCl prevented any binding of spin label I, indicating a denaturation of its binding site.

As pH is increased, the high spin iron of HRP is converted to low spin form and the enzyme no longer binds spin label I. From the changes in bound and free spin label intensity with variation of pH, the pK value of the acid alkali transition of HRP has been estimated to be 10.5 and is in good agreement with pK value of 10.8 obtained from other spectroscopic methods. As pH is decreased from 4.0 to 2.5, the intensity of high field component of rapidly tumbling spin increases considerably, indicating a substantial unfolding of apoprotein around its active site. The reversible and linear variation of $2T_{\shortparallel}$ with temperature changes would imply only a change in the rotational correlation time of the spin-labeled enzyme rather than any structural change.

On removing the heme cofactor from the enzyme, the apoHRP fails to bind the spin label. Recombination of apoHRP with manganic protoporphyrin IX, however, restored the peroxidase activity as well as its affinity for spin label I.

The fact that Zn(II) protoporphyrin IX-apoHRP compound did not bind the spin label I precluded its use as a diamagnetic control for detecting any interaction between the paramagnetic metals (Fe^{3+} or Mn^{3+}) and the spin label. The distance (r) between two spins held in a rigid matrix was calculated from the magnitude of their dipolar interaction given by

$$C = g\beta\mu^2\tau/r^6h$$

To insure rigid matrix environment for both free and bound spin labels, the ESR spectra were recorded at $77^{\circ}K$. From the ratio of amplitudes $\frac{A \text{ bound}}{A \text{ free}} =$ 90% found for both ferric and manganic HRP, the dipolar coupling constant was estimated and a lower limit of ~ 14 A° for the distance between the nitroxide group and the metal atoms was arrived at. This would support the model that the spin label is probably bound to HRP through polyfunctional hydrogen bonding rather than directly coordinating to iron.

Peroxidases perform important biological functions, e.g. catalysis of oxidation and halogenation of substrates in presence of toxic peroxides: The knowledge of active site topography in HRP is useful in understanding the structure-function relation of these enzymes.

The chain length of the spin label I will be increased by introducing glycine units between its aromatic and pyrrolidine rings. As the nitroxide moiety extends beyond the constrained hydrophobic crevice to more fluid environment, the rotational correlational time of the spin label will increase and the onset of its maximum value will help estimate size of the active site of HRP.

HRP forms a derivative with 2-naphthohydroxamic acid with an affinity one order of magnitude higher than that for spin label I. The study of such spin label analog might prove useful in probing the immediate environment of the heme group and may further our knowledge of active site conformation of HRP.

Publications: Wee, V.T., Feldmann, R.J., Tanis, R.J. and Chignell, C.F.: A comparative study of mammalian erythrocyte carbonic anhydrases employing spin-labeled analogues of inhibitory sulfonamides. Mol.Pharmac. 12: 832-843, 1976.

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PERIOD COVERED

July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)

Spin Label and Cu(II) Binding Studies of Serum Albumin

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

PI:	G. Rakshit	Visiting Fellow	PB NHLBI
	C. F. Chignell	Pharmacologist	PB NHLBI

COOPERATING UNITS (if any)

None

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The electron spin resonance (ESR) spectra of equimolar complexes of Cu(II) with plasma albumins from various species have been examined at 77°K. Below pH 7 canine and porcine albumin-Cu(II) complexes showed poorly resolved copper hyperfine splittings in contrast to four well-separated $g_{\parallel}$ lines observed in cupric derivatives of bovine and human albumins. At pH 11 nitrogen superhyperfine splittings were detected in all of the above complexes, indicating the involvement of three nitrogens in chelation with copper.

A topographic study of human serum albumin (HSA) has been initiated with the aid of a spin label analog of isothiocyanate. From its bound ESR spectrum, one strongly immobilized binding site ($2T_{\parallel}$ = 64 Gauss) has been detected. The effect of alterations in the conformation of HSA induced by changes in such parameters as pH have been examined.

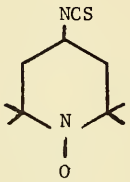
1203

Both spectral and chemical methods suggest that at neutral pH the first copper(II) atom is bound strongly to human and bovine serum albumins at a specific site presumably involving amino terminal, histidine and peptide nitrogens. Canine and porcine albumins, which lack this histidine residue at position 3, bind little Cu(II) at pH 7 and show no specificity. The aim of the present study is to understand the mechanism of copper binding to plasma albumins with the aid of the electron spin resonance (ESR) technique.

Crystalline preparations of human and bovine serum albumins were used as purchased. Canine and porcine albumins were purified by gel chromatography and then freeze dried.

From ESR spectra at 77°K only one cupric ion binding site was detected in human and bovine albumins. The $g_{//}$, $g_{\perp}$ and $A_{//}$ values (Table 1) of these complexes are characteristic of simple Cu(II)-peptide derivatives. At pH values below 7, equimolar addition of Cu(II) to canine and porcine albumins resulted in very poorly resolved hyperfine lines. This observation suggests that copper does not form well-defined complexes with these proteins. At pH 11, when maximum ionization of peptide nitrogens is observed, seven nitrogen superhyperfine splittings were detected in the $g_{\perp}$ line of Cu(II)-albumin complexes of all the above species examined.

The ESR spectrum of human serum albumin (HSA) labeled with an isothiocyanate spin label (I) was characteristic of a single highly immobilized nitroxide ($2T_{//}$ = 64 G). The spectral intensity of HSA-bound spin label remained essentially unaltered between pH 4 and 8. Below pH 4 the intensity of the rigidly bound spin label decreased at the expense of the freely tumbling form. This observation is consistent with the volume expansion of HSA associated with its N-F transition. Above pH 9 significant unfolding of HSA was indicated from the increased mobility of spin label. Reaction of HSA with either fluorodinitrobenzene or p-chloromercuribenzoate caused a reduction in labeling by I.



(I)

In hemolytic crisis of Wilson's disease, D-penicillamine is used as a chelator for excess Cu(II) deposited in body. However, this drug is not widely administered due to its lack of specificity and limited tolerance by patients. The knowledge of copper binding site in albumin is important for isolating a small analogous peptide which would be more specific in mobilizing excess copper.

Interaction of Cu(II) with alpha amino group will be studied using DNP-treated albumins. Binding of Cu(II) with Gly-Gly-His peptide will be investigated as a possible model for human serum albumin (HSA).

The possibility of interaction of isothiocyanate spin label with NH_2 terminal and free SH group of HSA will be explored by using group specific reagents.

Publications: None.

TABLE 1

$A_{\parallel}$ Value of Cupric Ion in Equimolar Complex of Proteins and Peptides

Ligand Compound	pH	$A_{\parallel}$ (in Gauss)
Gly-His-Gly	6.2	200
Human Serum Albumin	5.6	165
	10.2	198
Bovine Serum Albumin	5.5	165
	10.3	198
Dog Serum Albumin	10.6	196
Pig Serum Albumin	10.7	194

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HLB 02529-01 PB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Spin Label Studies of Prealbumin and Thyroxine Binding Globulin

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

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SECTION
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NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	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)

The present study was initiated to understand the mechanism of thyroxine binding by prealbumin (PA) and thyroxine binding globulin (TBG). When bound to PA, the spin label analogs of thyroxine and dansylsulfonamide became highly immobilized, indicating a constrained structure of its binding domain. The $2T_{\parallel}$ values of thyroxine spin label bound to PA and TBG were found to be 64 G and 45 G respectively. This observation suggests that the thyroxine binding site in TBG is less rigid than that in PA.

1206

Thyroxine binding globulin (TBG) and prealbumin (PA) are the major thyroxine transport proteins in human plasma. In contrast to limited data for TBG, the primary structure of PA is known and its amino acid sequence as well as its X-ray structure have recently been elucidated. While each of the four identical subunits of PA carries one retinol binding protein (RBP), the center of PA contains two symmetrically equivalent binding sites of thyroxine. The object of present study is to examine the topography of thyroxine binding domains of PA and TBG with the aid of spin label probes.

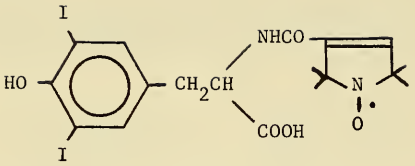
While diiodotyrosine spin label (I) did not bind PA, thyroxine spin label (II) was found to be highly immobilized upon binding to PA ($2T_{\prime\prime}$ = 64 G). When the second binding site of PA was also saturated with spin label (II), no dipolar interaction between the two bound spins could be detected. This would set the lower limit of distance between two nitroxide groups at 20 A^{o}, and it is consistent with X-ray data. The spin-labeled dansylsulfonamide analog (III), which competes with thyroxine for the same binding domain on PA, also became strongly immobilized on complexing with PA. The maximum hyperfine splitting of PA bound label III was found to be 65 G. The present study would indicate a rigid and constrained environment around the thyroxine binding site of PA.

Spin label I did not bind TBG while spin label II showed a strong immobilization upon binding to TBG. However, a smaller $2T_{\prime\prime}$ value (45 G) obtained for TBG-bound spin label (II) would indicate a less constrained environment of its binding site compared to that in PA. The fact that addition of paramagnetic salt $NiCl_2$ did not reduce the TBG-bound spin label (II) intensity would indicate that its binding domain is presumably buried inside the hydrophobic product, inaccessible to the aqueous phase. The treatment of TBG with 6 M guanidine HCl resulted in an enhanced mobility of bound spin label II, suggesting a substantial unfolding of its binding pocket caused by the denaturing agent.

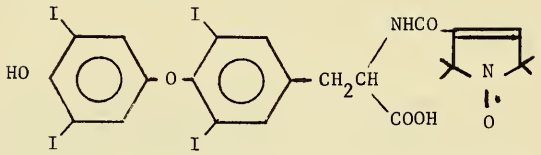
The knowledge of hormone binding sites in PA and TBG is important, since various drugs influence their hormone binding and may additionally affect the hormone metabolism.

Thyroxine analogs, in which the length of the side chain containing the spin label is increased, will be synthesized and used to study the topography of the thyroxine binding sites on PA and TBG. PA and TBG each contain one free sulfhydryl group per each monomer and chemical modification of this residue has been shown to influence thyroxine binding. Conformation of polypeptide chain around this cysteine molecule (not yet resolved by X-ray) will be examined with NEM spin labels of increasing chain length.

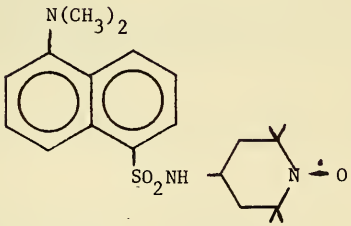
Publications: None.



(I)



(II)



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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HLB 02530-01 PB

PERIOD COVERED
July 1, 1976 to September 30, 1977

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

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SECTION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.5	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)

In collaborative studies with the Center for Ulcer Research and Education, V. A. Hospital, Los Angeles, the histamine-containing cell in dog gastric mucosa has been identified as a small mast cell with a histamine content of around 3 pg/cell. Histidine decarboxylase activity is also present in small amounts in this cell. The cell exhibits the characteristic metachromasia upon staining with toluidine blue and like the blood basophil is resistant to the histamine-depleting action of compound 48/80. Fractionation of other mucosal cells by centrifugal elutriation has further shown that the enzyme responsible for histamine degradation, histamine-N-methyltransferase (HNMT), is present in the parietal cell of dog mucosa. Additional studies with the new H_2 receptor agonist Dimaprit indicates that this compound has a similar affinity for HNMT and the H_2 receptor and is a noncompetitive inhibitor of the enzyme.

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Introduction: The introduction of the histamine H_2 receptor antagonists by Black and associates and the finding that these compounds effectively block gastric secretion in a variety of species provide convincing evidence that histamine has an essential role in gastric secretion. Many questions remain, however. It is not known where histamine is stored in the gastric mucosa, how it is released, or how it reaches the acid-secreting parietal cell. Nor do we understand the precise relationship of histamine to other mediators of gastric secretion. The question also arises as to how histamine is formed in sufficient quantities to participate in the regulation of gastric secretion. Except for rat, the enzyme responsible for histamine synthesis, histidine decarboxylase, is not present in high amounts in gastric mucosa.

The recent development of the technique of centrifugal elutriation for cell separation has led to the first successful separation of the different cell populations of the gastric mucosa into relatively pure fractions by Andrew Soll, Morton Grossman and associates at the Center for Ulcer Research and Education (CURE), Wadsworth V. A. Hospital, Los Angeles. In collaboration with this group, we have begun to investigate some of the questions concerning histamine's role in gastric secretion. In the initial stage of this work, we have identified the "histamine-containing cell" in the gastric mucosa and have determined the precise location of the enzymes responsible for histamine synthesis and metabolism in different mucosa cells. The second stage of this work will be concerned with the mechanism of histamine release and its interaction with histamine receptors on the parietal cell.

As a counterpart to these studies, we have undertaken a program to find more effective and specific inhibitors of histamine-N-methyltransferase (HNMT). Since HNMT is the only histamine-inactivating enzyme found in gastric mucosa of man and most other species, inhibitors of this enzyme would be useful in studies of histamine's role in gastric secretion.

Methods: Studies with Isolated Gastric Mucosal Cell. The work is divided into two phases. At CURE gastric mucosa is obtained from dog stomach and treated sequentially with a collagenase preparation, EDTA and then a second collagenase treatment. At all stages of the work, cell viability is monitored by microscopy, dye exclusion tests. The cells are harvested from the disrupted mucosa by filtration and washed. The cells are then subjected to a preliminary separation in a Sorvall zonal rotor using an albumin density gradient. This procedure separates the cells into a histamine, parietal, or chief cell-enriched fraction. Each of these is further fractionated according to cell size on the Beckman elutriator. The fractions are divided into aliquots of about 10^6 cells, and duplicate sets are shipped to the NIH. Apart from electron microscopic examination (Dr. Juan Lechago, Harbor General Hospital, Los Angeles), the parameters measured at CURE include: standard biochemical markers (DNA and protein); specific cell markers (succinic dehydrogenase for parietal cell, glucagon for the enterochromaffin cells and pepsinogen for the chief cells); cell count; cell size analysis; and response to physiological stimulants (acetylcholine, histamine, gastrin) as measured by "O" consumption and cyclic AMP production.

At the NIH the different fractions are assayed for their histamine and serotonin content, histidine and DOPA decarboxylase activity, and HNMT and diamine oxidase activities using the micro-assays developed in this laboratory. These assays have been described in previous annual reports, although further refinement of some procedures has been necessary because of the small size of the aliquots. Assays have been performed by the principal investigator on fresh samples at CURE and on frozen samples shipped to NIH.

Studies with Inhibitors of HNMT. The sources of enzymes were as follows. For histidine decarboxylase, a soluble extract of rat gastric mucosa was used. A soluble extract was prepared likewise from rat ileum as the source of DAO. A partially purified preparation of HNMT was prepared from frozen guinea pig brain. Histidine decarboxylase activity was assayed by measurement of the $^{14}CO_2$ release from L-histidine-(^{14}C-carboxyl carbon), DAO activity by measurement of tritium release from β-^{3}N-histamine and HNMT by the formation of ^{14}C-methylhistamine from histamine in the presence of S-adenosyl-L-methionine (^{14}C-methyl).

Results: Distribution of Histamine, Serotonin and their Metabolizing Enzymes in the Gastric Mucosal Cell Fractions. Profiles of the different cell fractions in 3 experiments have shown that histamine is located exclusively in a narrow band of small sized cells (9-9.5 μ diameter), which have under electron microscopy the characteristic morphology of mast cells. Initially, these cells were difficult to identify microscopically until it was discovered that cell structure was preserved only with glutaraldehyde fixation. The cells show characteristic metachromasia with toluidine blue. The histamine content of the most enriched fraction of these cells was 2.9 pg/cell (cf. the basophil 1-1.5 pg/cell). Preliminary studies indicate that the amine is released (∿ 10%) in the presence of carbachol, gastrin and PGE_1 but not by compound 48/80.

Profiles of histidine decarboxylase activity indicate that the enzyme is present in the histamine-containing cells. The enzyme has the characteristics of the specific histidine decarboxylase. The activity, however, is low and is sufficient to account for only a slow turnover of histamine in these cells. Studies with partially purified histidine decarboxylase activity from rat stomach give no indication that inhibitors of the enzyme (at least to the rat enzyme) are present in the mucosal cells.

The profile of HNMT activity paralleled that of parietal cell, although some activity appeared to be present in other cells. The enzyme activity was high and was more active (when expressed as units/mg protein) than our partially purified HNMT preparations from guinea pig brain. The histamine-containing cells were virtually devoid of HNMT activity. Diamine oxidase activity could not be detected in any cell fraction.

Serotonin was present in band of large cells (12-13 μ) which have been identified as the larger of two populations of chromaffin cells found in the gastric mucosa.

Studies with HNMT Inhibitors. HNMT is inhibited with high concentrations ($\sim 10^{-5}$ M) of substrate and by the H_2 receptor antagonist, burimamide. For this reason, we are screening compounds which interact with histamine-H_2 receptors as possible inhibitors of this enzyme. Recently, Dimaprit, S-[3-(N, N-dimethylamino)propyl]isothiourea, was introduced as a selective agonist for histamine H_2 receptors. The compound possesses less than 0.001% of the activity of histamine on H_1 receptors (guinea pig ileum) and is highly active towards tissues with H_2 receptors (rat uterus, guinea pig right atrium and gastric mucosa) (Parsons et al., AgentsandActions 7: 31, 1977). When the effect of Dimaprit on HNMT and on other enzymes involved in histamine metabolism was examined, the following results were obtained.

TABLE 1

Percent Inhibition of Histamine-metabolizing Enzymes by Dimaprit in vitro

Dimaprit conc. (M)	Histidine decarboxylase (rat gastric mucosa)	Diamine oxidase (rat ileum)	Histamine-N-methyltransferase (guinea pig brain)
10^{-6}	0	1	7
10^{-5}	0	3	56
10^{-4}	0	14	89
10^{-3}	2	70	90
2.5×10^{-3}	1	81	95

The values are the mean of two assays.

Dimaprit was found to inhibit HNMT and, in higher concentrations, DAO. The compound had no effect on histidine decarboxylase activity (Table 1). The inhibition of both HNMT and DAO was noncompetitive. In these and 2 other experiments, the K_i values as determined by the Dixon plot ranged from 7-9 x 10^{-6} M for HNMT and 2.5-3.0 x 10^{-4} M for DAO. The affinity for HNMT is similar to that reported for the H_2 receptor in atrium and the uterus (Black et al., Nature 236: 385, 1972). In comparison with other published K_i values, Dimaprit was more effective an inhibitor of HNMT than the majority of the antihistaminic compounds tested by Taylor and Snyder (Mol.Pharmac. 8: 300, 1972), more effective than the H_2 antagonist burimamide (K_i 2.8 x 10^{-4} M) (Taylor, Biochem.Pharmac. 22: 2775, 1973), but less effective than the antimalarial drug Quinacrine (K_i 1 x 10^{-7}).

HNMT is also inhibited the reaction product 1-methylhistamine. The inhibition by methylhistamine (K_i 2-4 x 10^{-4} M) appeared to be competitive but at

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higher substrate concentrations (5×10^{-6} M) noncompetitive. At high substrate concentrations, the inhibition of HNMT by Dimaprit and methylhistamine was enhanced. When Dimaprit and 1-methylhistamine were tested in combination, the K_i value for Dimaprit was unaltered by the presence of 1-methylhistamine and vice versa.

Significance to Biomedical Research and Future Studies: The studies with isolated gastric mucosal cells have led to the identification of the cellular source of histamine in mucosa as a "mast cell" type of cell. Histidine decarboxylase has been identified in these cells. Although the activity is low, this is the first direct demonstration of this enzyme in dog gastric mucosa. High levels of histamine-N-methyltranferase, on the other hand, are associated with the parietal cells. Thus the target H_2 receptor for histamine and the enzyme responsible for its inactivation may be present in the same cell. Of interest is the finding that an agent with a selective affinity for the H_2 receptor, Dimaprit, also has a similar affinity for HNMT and is a noncompetitive inhibitor of the enzyme.

Future studies will be concerned with the properties of the histamine stores in the gastric mucosal cells and the responsiveness of these cells to known stimulants, gastrin, cholinergic agents and compound 48/80. The possibility that histidine decarboxylase activity is induced in these cells by gastrin will also be examined. More detailed biochemical examination of these cells (e.g. activation of adenylate cyclase) will follow once the basic receptors on the cells have been identified.

The HNMT inhibitory activity of other compunds related to Dimaprit will be examined.

Publications:

Shaff, R.E. and Beaven, M.A.: Inhibition of histamine-N-methyltransferase and histamine (diamine oxidase) by a new histamine H_2-agonist, Dimaprit. Biochem.Pharmac., in press.

Shaff, R.E. and Beaven, M.A.: Turnover and synthesis of diamine oxidase (DAO) in rat tissues. Studies with heparin and cycloheximide. Biochem.Pharmac. 25: 1057-1062, 1976.

Beaven, M.A.: Histamine and its participation in dermatological disorders, Part 1. Progr.Dermatol. 11: 15-22, 1977.

Beaven, M.A.: Histamine and its participation in dermatological disorders, Part 2. Progr.Dermatol., in press.

Moss, J., de Mello, M.C., Vaughan, M. and Beaven, M.A.: Effect of salicylates on histamine and L-histidine metabolism. Inhibition of imidazoleacetate phosphoribosyl transferase. J.Clin.Invest. 58: 137-141, 1976.

Beaven, M.A., Horakova, Z. and Keiser, H.R.: Interference with histamine and imidazole acetic acid metabolism by salicylates: A possible contribution to salicylate analgesic activity? Experientia 32: 1180-1182, 1976.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HLB 02531-01 PB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Carrageenan-induced Inflammation: Studies of the Action of Anti-inflammatory Drugs

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

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	M. A. Beaven	Pharmacologist	PB NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Pulmonary

SECTION
Molecular Pharmacology

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.3	0.1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Carrageenan-induced inflammation in rat pleural cavity was used as a model to study how cellular activity is altered by anti-inflammatory drugs during the inflammatory response. In the early stages of the response, the pleural exudate contained large numbers of intact basophils and had high levels of histamine which were located almost entirely in the cells. At later stages, the histamine content and basophil count decreased and the exudate consisted largely of neutrophils. Pretreatment with H_1 and H_2 antihistaminic drugs produced a partial (42%) reduction in fluid volume, as did pretreatment with the histamine liberator compound 48/80. Anti-inflammatory drugs, in pharmacological doses, produced a marked decrease in volume (63-73%) as well as in the number of cells mobilized. The histamine content of total exudate was not significantly altered by this treatment. These results suggest that histamine plays only a minor role in this form of inflammation and that the major effect of treatment with anti-inflammatory drugs is a reduction in volume and the number of cells mobilized.

1214

Introduction: Because of the inhibitory effects of the anti-inflammatory drugs on cell cultures (described in Report No. Z01 HLB 02520-02 PB), a program was started to investigate the effects of these drugs on cellular function in inflammatory exudates. Studies by Vinegar and associates (Fed.Proc. 35: 2447, 1977) have shown that carrageenan will induce a nonchronic "inflammatory reaction" in the rat paw or pleural cavity. The "inflammatory" reaction increases gradually, reaches a maximum at 4 hr and then slowly subsides over the course of 24 hr. The reaction is characterized by an accumulation of an extravascular exudate which contains both lymph and blood derived cells. The cellular elements (predominantly the polymorphonuclear cells) involved are similar to those seen in other inflammatory reactions. Both the volume of exudate and the numbers of cells mobilized are markedly suppressed by the anti-inflammatory drugs. For this reason, we have adopted the carrageenan model for our in vivo studies.

In the initial phase of our studies, we are attempting to characterize the carrageenan reaction in more detail, particularly the identification of 1) the cells involved and 2) cellular factors which are secreted during the reaction. The factors include known inflammatory mediators such as histamine, serotonin and the prostaglandins; chemotactic factor(s) and possibly other factors which could be used as markers of cellular activity. In later stages of the study, we will attempt to determine whether the anti-inflammatory drugs inhibit specific stages of cellular activity, e.g. mobilization, chemotaxis, phagocytosis and excretion of inflammatory and chemotactic factors.

Procedures: Inflammation was induced in the pleural cavity of Sprague-Dawley rats ($\sim$ 300 g) by injection of 500 µg carrageenan. The fluid exudate was collected after various time intervals by anesthetizing rats with ether, by aspiration into hypodermic syringes. Volume of the exudate was noted. Cell count was determined by dilution of 10 µl of fluid into 1 ml of Isoton and counting in a Neubauer counting chamber. Differential cell counts were also determined. The remainder of the fluid was frozen for the assay of the various mediators.

Anti-inflammatory drugs were administered orally as suspensions in cornstarch gel. Other drugs were injected intraperitoneally as saline solution.

Histamine and serotonin were assayed by the enzymatic isotopic techniques described in previous annual reports.

Results: Time Course of Response to Carrageenan. At 1 hr small amounts of a viscous white fluid, 0.1-0.2 ml, had collected in the pleural cavity. The exudate contained a surprisingly large number of mast cells (15%) and eosinophils (13%). No disrupted mast cells could be seen microscopically. Over the period of 2-4 hr, fluid volume increased markedly and at 4 hr volumes of 1-2 ml were recovered from most rats. At this time, the exudate consisted almost entirely of neutrophils ($\sim$ 90%). The cell count increased from around 40×10^6/ml at 1 hr to 150-200 $\times 10^6$/ml at 4 hr. At this time, the basophils accounted for less than 5% of the cells.

High histamine levels (71 ± 8 µg/ml, n = 9) were found in the exudate at 1 hr. By 4 hr, the levels had declined to 12 ± 2 µg/ml (n = 9). This decline was consistent with the decrease in basophil count. Almost all (> 99% of the histamine was located in the cell. The levels of histamine in the extracellular exudate was 62 ± 7 ng/ml at 4 hr. Serotonin was also present in the cellular fraction but at much lower concentrations, 0.4 ± 0.1 µg/ml (n = 8), than histamine. Prostaglandins have not been measured.

Effect of Anti-inflammatory and Antihistamine Drugs on Exudate Production. As measured at the 4-hr time period, the accumulation of fluid was reduced by pretreatment with aspirin, 100 mg/kg (73 ± 17%), phenylbutazone 200 mg/kg (73 ± 15%) and indomethacin 5 mg/kg (63 ± 13%).

In general, histamine levels increased (as compared to controls), but the histamine content of the total exudate was not significantly altered. This result suggests that the total number of mast cells "mobilized" had not been affected by the drugs. Differential cell counts have not been undertaken at this time.

Pretreatment with the histamine H_2-receptor antagonist, metiamide, and the H_1-receptor antagonist, mepyrine (50 mg/kg), had little effect on fluid volume. Pretreatment with higher doses (100 mg/kg) of mepyramine reduced the volume by 38 ± 7%, and pretreatment with both antagonists (100 + 100 mg/kg) resulted in a modest but significant reduction of exudate (42 ± 9%). Pretreatment with the mast cell depletor, compound 48/80. has been reported to reduce greatly the response to carrageenan. In our hands, such treatment resulted in a reduction in fluid volume (28 ± 9%) when the drug was administered daily for 4 days prior to inoculation and a larger reduction (70%) when administered the previous day and 2 hr before inoculation with carrageenan.

Significance to Biomedical Research: The nonsteroidal anti-inflammatory drugs have less effect on the initial inflammatory response to injury but have a significant effect on later stages of the response. This later stage is characterized by the accumulation of cells. Since carrageenan administration reproduces the responses seen in these later stages of acute inflammation, it is hoped that this will provide a useful model to study the types of cellular activity influenced by the anti-inflammatory drugs.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HLB 02532-01 PB

PERIOD COVERED
July 1, 1976 to September 30, 1977

TITLE OF PROJECT (80 characters or less)
Relationship Between Mitogenic Transformation and $(Na^+ + K^+)$-ATPase

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

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OTHER:	E. O. Titus	Head, Section on Molecular Pharmacology	PB NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Pulmonary

SECTION
Molecular Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	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)

Although concanavalin A and other lectins inhibit purified $(Na^+ + K^+)$-ATPase, an enzyme involved in cation transport, concanavalin A stimulates the enzyme when incubated with intact thymocytes. The latter may be a useful model for studying physiological regulation of membrane transport.

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Project Description:

Objectives: An early step in immune response is the blastogenic transformation of lymphocytes. This transformation can be artificially induced by various lectins. Induction is accompanied by a rapid increase in the active transport of potassium. All of the sequelae to transformation, including mitosis, increased respiration and synthesis of protein, DNA and RNA are dependent on the increased ouabain-sensitive potassium influx. It seems likely that activation of ($Na^+ + K^+$)-ATPase occurs very early in transformation, irrespective of whether it is itself the initiating event.

Since the induction of lymphocyte ATPase by lectins apparently does not involve new protein synthesis and since lectins are reported to inhibit rather than to activate isolated transport ATPase, it is possible that lectins act by altering the association of the enzyme with endogenous activators or inhibitors in the intact membrane. A promising candidate for such a regulatory substance would be the glycoprotein which is invariably associated with the larger catalytically active component in purified preparations. It has been reported that removal of this protein can cause remarkable increases in both the activity and lability of a brain ($Na^+ + K^+$)-ATPase. Bridging by divalent lectins of this glycoprotein to mobile components of the cell membrane would offer a means of regulating ATPase that is consistent with the known effects of lectins on cell surface glycoproteins. If the activation of ATPase indeed occurs by lateral separation of regulator from effector, the process should be inhibitable by inactivators of the tubulin system, such as colchicine.

The immediate purpose of this project is to find lymphocyte systems in which the transport ATPase can be stimulated by lectins and to test the possibility that release from inhibition by an endogenous glycoprotein is the stimulating event. In view of published reports that con A releases small glycoproteins from rabbit thymocytes, some of the preliminary work was done with these cells.

Methods: Lymphocytes were prepared by expression from rabbit spleen and thymus through fine screens. Washed cells or microsomal fractions prepared from cell homogenates were incubated with ATP, Mg^{++}, Na^+ and K^+ in the presence and absence of ouabain, the difference in yield of inorganic phosphate being taken as a measure of ($Na^+ + K^+$)-ATPase activity.

Purified ATPase was prepared from rabbit kidney microsomes by Jørgensen's (Methods in Enzymology 32: 277, 1974) procedure.

Major Findings: Concanavalin A (con A), phytohemaglutinin and lectins from wheat germ and lentil inhibited purified ($Na^+ + K^+$)-ATPase. Inhibition did not exceed 25-30%, even as quantities of lectin considerably in excess of the ATPase protein were introduced. Since con A is known to require Ca^{++} and Mn^{++} for its characteristic binding to α-glucosides and mannosides, ATPase was incubated with con A, with and without 0.5 mM concentrations of these ions. After washing away of ions and unbound lectin, control samples were found to

retain 1.47 mg of con A per mg of ATPase protein, with negligible inhibition. Ion-treated samples retained 1.64 mg/mg with 41% inhibition. Exposure to ion alone did not inhibit the ATPase, and increases in ion concentration did not further increase the inhibitory effects of con A.

After incubation for 20 hours in Grand Island RPML-1640 media with HEPES, glutamine, antibiotics and 10% calf serum, thymocytis from 10- to 13-week old rabbits contained measurable levels of both Mg^{++}-ATPase and $(Na^{+} + K^{+})$-ATPase and became sensitive to con A. Enhancement of these activities by incubation for 2 hours was optional at 10-15 g of con A per ml. Rabbit spleen lymphocytes were obtainable in poor yield and difficult to purify without loss of viability, but exhibited similar sensitivity to con A.

Levels of ATPase in lymphocytes after 2 hours incubation with con A

		nmoles/mg protein per minute	
	Additions	$(Na^{+} + K^{+})$-ATPase	Mg^{++}-ATPase
Spleen	None	6.0	160
	15 µg con A/ml	8.6	207
Thymus	None	7	69
	10-15 µg con A/ml	9.9	83.3

Significance to the Program of the Institute: Changes in cellular levels of transport ATPase are known to occur in response to insulin, catecholamines, oncogenic and mitogenic transformation and other stimuli. Although a number of mechanisms, including altered levels of calcium and cyclic nucleotides, have been postulated, the mechanism by which ion transport accommodates to altered physiological status remains obscure.

Proposed Course of Project: The project will end upon dissolution of the Section on Molecular Pharmacology.

Publications: None.