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OF
PROGRAM ACTIVITIES
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
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ANNUAL REPORT of the director

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NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
Annual Report
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Office of the Director

Under its congressional mandate, the National Heart, Lung, and Blood Institute

--conducts and supports fundamental research on the heart, blood vessels, blood, and lungs and on the diseases that afflict them;

--develops and evaluates new or improved means for the prevention, detection, diagnostic evaluation, and treatment of these diseases;

--encourages widespread application of proven new techniques by the research and medical communities;

--provides support for training research workers, clinicians, and teachers in the cardiovascular, blood, and pulmonary fields; and

--informs the general public and health professionals about research and clinical advances arising from Institute programs.

Some highlights of progress during the year in major NHLBI program areas are briefly described in the sections that follow. Most of the work cited was conducted or supported by NHLBI.

HEART AND BLOOD VESSEL DISEASES

Primary Prevention

The ultimate objective of Institute research is the prevention of disease. Successes to date have come about through identification of factors that increase susceptibility to heart and blood vessel diseases as well as evaluation and application of countermeasures against modifiable risk factors thought to be involved in the causation of disease. With respect to the major cardiovascular diseases within the purview of the Institute, the three most important modifiable risk factors are cigarette smoking, elevated blood pressure and elevated lipids.

Elevated blood cholesterol is sometimes hereditary (familial Type II hyperlipoproteinemia in the NIH classification system). Among Type II homozygotes, cholesterol levels are grossly elevated from birth and clinical manifestations of premature arteriosclerosis may become evident during childhood or adolescence. Among Type II heterozygotes, the cholesterol elevations, though less marked, are still substantial, and heart attacks before age 50 are quite common among untreated subjects. Attempts to normalize blood cholesterol in Type II homozygotes have been unsuccessful, but heterozygotes may be managed successfully with therapeutic diets supplemented with cholesterol-lowering drugs. Studies are now suggesting that the combined regimen may retard the progression of arteriosclerosis.

In a recently reported study among 32 Type II heterozygotes, total cholesterol levels averaged 348 mg/dl and LDL cholesterol averaged 304.5 mg/dl at entry. The therapeutic diet alone reduced these levels by 12.8% and 23.1% respectively. When the diet was supplemented with 30 mg. daily of the cholesterol-lowering drug colestipol, total cholesterol fell by an additional 17.5% and LDL cholesterol fell by 23.1%. When nicotinic acid (1.5 gm. daily) was then added to the regimen, total cholesterol fell by 42.6% and LDL cholesterol by 52.1%, bringing these values into the normal range in most subjects. Also, on the combined regimen, the subjects' triglyceride levels fell by 45.4% and their HDL cholesterol (thought to be protective against atherosclerosis) rose by 20.4%.

Twenty-eight of the subjects had undergone coronary angiography at the start of the study because of coronary heart disease (CHD) symptoms. Repeat angiograms were done in 16 patients after three or more years on the diet-plus-drugs regimen. Twelve subjects, whose response to the regimen had been good, showed no progression of earlier lesions nor were there any new ones. Though no discernible regression of pre-existing lesions was observed, the combined regimen had apparently halted the disease in its tracks. However, in four patients who had responded poorly to the regimen, in terms of failing to achieve substantial blood cholesterol decreases, variable degrees of progression of their CHD were observed.

The elevated blood cholesterol levels of familial Type II hyperlipoproteinemia are thought to result from a genetic deficiency of LDL receptors in the cells of liver and other tissues. These receptors are involved in the removal of LDL cholesterol from the plasma for use by the cells in new membrane synthesis or for other metabolic purposes. The number of available LDL receptors determines at what level of plasma cholesterol uptake by the tissues will occur. In Type II heterozygotes only about half the normal number of LDL receptors are available, and the body compensates by doubling the plasma cholesterol. The source of the extra cholesterol is the liver, which steps up production by increasing the activity of HMG CoA reductase, the enzyme that presides over the rate-limiting step in cholesterol synthesis.

A number of cholesterol-lowering drugs stimulate the production of LDL receptors, thereby increasing the rate of cholesterol catabolism. However, in Type II heterozygotes, the more limited increase in LDL receptors is partially offset by increased cholesterol synthesis by the liver. But two new agents called compactin and mevalonin, isolated from a penicillin mold by Japanese and American pharmaceutical companies, exhibit a dual action that may make them especially useful in the treatment of familial Type II hyperlipoproteinemia. Both drugs increase the production of LDL receptors, thereby increasing the uptake and catabolism of plasma cholesterol; but both also inhibit liver HMG CoA reductase thereby blocking any compensatory increase in cholesterol synthesis.

Mevalonin, the more potent of the two, has thus far been tested only in animals. Studies in dogs showed that the drug decreased LDL cholesterol synthesis by 50%. When mavalonin was given to the animals along with colestipol, another cholesterol-lowering agent, the combination produced a threefold increase in LDL receptors and reduced plasma LDL cholesterol by an amazing 75%.

Compactin has undergone very limited clinical testing in Japan. In one recently reported study among Type II heterozygotes, small daily doses of compactin (30-50 mg.) reduced mean plasma cholesterol levels by 22%. Plasma triglyceride levels also fell significantly. The decrease in blood lipids was achieved chiefly at the expense of the atherogenic LDL fraction. The apparently protective HDL fraction was little affected by the drug. And in an earlier study among Type II heterozygotes and homozygotes, 50-150 mg. of compactin a day reduced plasma cholesterol levels by an average of 27%.

No adverse drug effects were observed in these limited trials; however, no results from long-term testing for toxicity in animals or humans have been reported, and so there remains some possibility of unexpected side effects. But for now both agents hold unusual promise because they lower blood cholesterol substantially, and without impeding the delivery of needed cholesterol to tissues or depleting bodily stores of this vital sterol.

Other studies have examined various dietary supplements as possible means of treating patients who respond poorly to conventional cholesterol-lowering diets, find them difficult to follow, or find cholesterol-lowering drugs troublesome or inconvenient.

One supplement that holds some promise is a synthetic, non-absorbable fat called sucrose polyester (SPE). In the diet SPE can be readily substituted for conventional vegetable oils and shortenings, since it can be made to resemble them in consistency, taste, and other characteristics important in cookery. But when consumed, SPE cannot be absorbed from the gut. Moreover, SPE has a high affinity both for cholesterol and for certain of its derivatives produced by bacterial action in the gut. Thus, during its passage through the gut, SPE binds much of the cholesterol consumed along with it as well as any other cholesterol or conversion products encountered along the way. Cholesterol or its derivatives bound by SPE are subsequently excreted with it.

It is primarily by impeding the absorption and increasing the excretion of dietary cholesterol that SPE affects blood cholesterol levels, though it can also bind endogenously produced cholesterol secreted into the gut and normally reabsorbed via the enterohepatic pathway. SPE apparently does not affect the absorption of nutrients other than cholesterol nor does it affect the turnover of bile acids, the usual pathway of cholesterol excretion. The agent is most likely to be useful in those subjects who are hyperresponsive to dietary cholesterol. Also because SPE is non-nutritional, its substitution for conventional sources of dietary fat may make it useful in weight-reduction regimens.

Another interesting supplement that has undergone very limited testing is locust bean gum (LBG), a tasteless, colorless, and odorless extract from the fruit of the honey locust tree. The sticky texture of LBG posed some problems, but it seems to be easily disguised in baked goods such as cookies, crackers, and breads. Such LBG-fortified foods were

pronounced "tasty" by 28 hypercholesterolemic subjects who, over an 8-week period, consumed 8-30 grams of them daily as supplements to a conventional cholesterol-lowering diet. In these subjects, the LBG supplement produced a 20% greater reduction in cholesterol than that achieved with the unsupplemented diet while causing no significant side effects. These results compared favorably with those of diets supplemented with cholesterol-lowering drugs, which cause troublesome side effects in many subjects.

Another investigator, noting that alfalfa meal supplements to an atherogenic diet caused regression of atherosclerotic deposits in the aortas and coronary arteries of monkeys, cautiously tested ground, roasted alfalfa seeds as a dietary supplement in 13 patients with hypercholesterolemia. The patients consumed either 80 gm. or 100 gm. of the seeds daily (mixed with juice or other liquids) in addition to their "normal" diets. Among those consuming 80 gm. daily, blood cholesterol levels fell by 12% and other blood lipids remained unchanged; but among those consuming 100 gm of the seeds daily, cholesterol levels fell by 25% and triglycerides fell by 43%. The investigator believes that alfalfa seeds may lower cholesterol levels by interfering with the absorption of cholesterol and perhaps other dietary lipids and by increasing the excretion of bile acids. He points out that there may be problems of toxicity that did not show up in the earlier short-term studies and that if alfalfa seeds do, in fact, increase bile acid excretion, this might conceivably produce harmful affects on the mucosa of the colon and possibly predispose to colonic cancer. Further animal studies looking into these questions are presently in progress.

<u>The Multiple Risk Factor Intervention Trial</u> (MRFIT), begun in 1973, involves nearly 13,000 men at increased risk from coronary heart disease (CHD) because of various combinations of three major risk factors: elevated blood cholesterol, elevated blood pressure, and cigarette smoking. Any one of these factors roughly doubles CHD risk and the presence of all three can increase it by a factor of ten or more.

All three are modifiable, and the aim of the MRFIT study has been to intervene against all three in a special-care group and to compare the resultant effect on cardiovascular morbidity and mortality in this group with that of control subjects who had been referred to their usual sources of medical care.

At the four-year followup mark, study data indicated that the special-care group had reduced its average cholesterol level from 254 mg/dl to 235 (about 7%); average blood pressure from 93 to 82 mm/Hg (about 12%); and, most remarkably, 41% of the group had quit smoking. However, the controls had also done quite well on their own: their average cholesterol level was down by 4.4%, their average blood pressure had been reduced by 6.4%, and 23% had quit smoking. Though the CHD risk of the special-care group is still estimated to be about 40% lower than that of the controls, the risk-reduction measures which many men in the control group have taken on their own may diminish the expected between-group differences in CVD morbidity and mortality.

However, the results to date also suggest that large numbers of Americans may be voluntarily modifying lifestyles and following the advice of their physicians on matters affecting cardiovascular-disease risk. This could well be a significant factor in the gratifying reductions in mortality from coronary heart disease and stroke that have occurred in the U.S. during recent years.

Hydrochlorothiazide and chlorthalidone, diuretics widely used in the treatment of mild hypertension or as part of combination drug regimens for treating severer stages of the disease, produce increases in blood lipids that could partially negate the decrease in CHD risk attending adequate blood pressure control. In a study among male subjects with diastolic pressures of 85-105 mm/Hg, daily doses of 100 mg. hydrochlorothiazide or chlorthalidone raised total blood cholesterol by 14.3 mg/dl and LDL cholesterol by 18.8 mg/dl, respectively, as compared with placebo-treated controls. There were also significant drug-induced increases in plasma triglyceride levels.

However, among subjects receiving chlorthalidone, but who were also placed on the AHA Prudent Diet during the period of study, there were no significant increases in plasma cholesterol levels, nor did plasma triglycerides increase as much as among subjects receiving diuretics only.

The investigators feel that drug-induced increases in total cholesterol (which averaged 8% in this study) could partially cancel any reduction in CHD risk resulting from the modest decreases in systolic blood pressure (averaging 5% in this study) resulting from diuretic therapy, unless physicians prescribing those agents for mild hypertension also check their patients' serum lipids periodically and recommend dietary modifications if indicated.

Does vasectomy, a sterilization procedure undergone by some 250,000 U.S. males each year, increase susceptibility to atherosclerosis? A series of studies in primates has suggested that it may: the investigators noted that vasectomized monkeys developed more extensive atherosclerosis on comparable diets than did sham-operated but non-vasectomized controls. Their earlier studies involved only a few animals, but subsequent studies in 150 monkeys produced generally similar findings: on an atherogenic diet, vasectomized animals again exhibited more extensive atherosclerosis at autopsy than did controls, the differences between groups being most pronounced among those animals developing the highest cholesterol levels on the diet.

The investigators feel that, in the presence of other risk factors, such as elevated blood lipids, vasectomy may potentiate the development of atherosclerosis. One postulated mechanism is as follows: Vasectomy does not affect sperm production, but blocks their egress from the body. Trapped sperm subsequently break down and their components leak into surrounding tissues, gaining access to the blood. There, because mature sperm were not present during early life when the immune system was learning to distinguish between self and non-self, certain sperm proteins may be identified by this system as "foreign," thus stimulating the formation of

immune complexes. These, in turn, may activate components of complement that can damage the endothelium of the artery wall and promote the deposition there of plasma lipids.

Thus far, however, these findings in primates have not been shown to apply to humans. One study, comparing the incidence of non-fatal heart attacks among vasectomized and non-vasectomized males matched according to age and period of observation, revealed no significant differences between the two groups. Another compared hospitalization rates for various diseases--including circulatory disorders and acute heart attacks--among vasectomized vs. non-vasectomized males enrolled in a large group health plan. Again, with the single exception of genitourinary problems (for which hospitalizations were more frequent in the early period following vasectomies) there were no significant differences between groups.

The studies involved large numbers of subjects, but relatively few of the vasectomized males had undergone the procedure 10 or more years ago. Since atherosclerosis usually has a long latency period before its clinical manifestations become evident, the relative scarcity of long-term data might have tended to conceal any adverse effects of vasectomy on the cardiovascular system. Additional long-term data should be forthcoming from other studies presently underway with funding from NICHD.

Among women who use oral contraceptives (usually an estrogen combined with a progesterone type drug), the risk of heart attacks is three to four times that of non-users. Though heart-attack risk diminishes soon after discontinuing the pill and continues to decrease with time, some excess risk may persist for 10 years or more afterward, the results of a recent NICHD study indicate. The magnitude of the residual risk varies with the duration of use prior to discontinuing the pill and the time since cessation: the longer the initial period of use and the shorter the time since cessation, the greater the residual risk.

However, this excess heart-attack risk apparently does not apply to postmenopausal women taking female hormones (usually limited to estrogen type drugs without progesterone), for the relief of menopausal symptoms, another study indicates. In fact, among younger women (aged 33-45) who had experienced early menopause or women who had undergone bilateral oophorectomy (surgical menopause), use of the hormones reduced heart-attack risk by 40-60% as compared with that of otherwise similar non-users.

In general, relative risk was lower among current users than among past users, but both had a 10-30% lower heart-attack risk than did women who had never used the hormones. The duration of hormone use was not a significant factor in assessing relative risk and, surprisingly, neither was a history of cigarette smoking, which substantially increases the risk of cardiovascular complications, particularly stroke, among oral contraceptive users.

In deciding whether to prescribe hormone replacement in postmenopausal women, the physician must weigh potential cardiovascular or other benefits against known or suspected risks from other diseases, such as endometrial or uterine cancer in women with intact uteri. However, the study results indicate that patients with early or surgical menopause stand to benefit substantially from hormone therapy.

This finding may be of particular importance, since the results of another study among women less than 56 years old indicates that surgical menopause causes a nearly threefold increase in heart-attack risk as compared with that of premenopausal women. The younger the woman when bilateral oophorectomy was performed, the greater the risk: for women undergoing such surgery before age 35, the heart-attack risk was 7.2 times that of premenopausal women. Natural menopause before age 35 was associated with some increase in risk, but not nearly so great as that resulting from early bilateral oophorectomy.

It has been estimated that about one-fourth of women under 40 who undergo hysterectomy also have both their ovaries removed. In this study hysterectomy *per se* had very little effect on heart-attack risk, provided that one or both ovaries were left intact. If both ovaries are taken along with the uterus, the heart-attack threat appears to be real, and may be ameliorated by hormone replacement therapy.

Primary prevention is, of course, the most cost effective approach to nearly any disease problem. This is especially so with respect to heart and blood vessel diseases, whose initial clinical manifestations are so frequently lethal or permanently disabling. But even should the risk reduction measures previously described live up fully to the promise they seem to hold, we would still have a very large population of Americans in whom such measures have come too late.

Secondary Prevention

Several NHLBI-supported clinical trials are concerned with secondary prevention. They are evaluating measures that show promise in reducing disability or improving long-term survival among persons already stricken by clinical manifestations of arteriosclerosis.

The *Beta-Blocker Heart Attack Trial* (BHAT), begun in 1977, involved nearly 4,000 men and women who had sustained documented heart attacks immediately prior to entry into the study and had no contraindication to the use of beta blockers. These subjects were randomly assigned either to long-term treatment with propranolol or to a placebo-treated control group. Propranolol, a beta adrenergic blocking agent, slows heart rates, reduces blood pressure, reduces the cardiac workload, and confers protection against certain types of cardiac arrhythmias. It was believed that these potentially beneficial effects might result in reduction of the incidence of recurrent heart attacks and sudden cardiac death and would reduce overall cardiovascular mortality among drug-treated patients.

This, in fact, proved to be the case. Among drug-treated patients, the average treatment period was 24 months (range: 12-37 months). Overall mortality was 7% in this group versus 9.5% in the control group--a 25% reduction in mortality among drug-treated patients. Moreover, in each subgroup studied, the benefits of propranolol therapy pertained independently of patients' age, sex, race, part of the heart involved, or apparent severity of the heart attack. The benefits of propranolol therapy appeared greatest during the first 12-18 months, but this is also the period of greatest risk for the MI survivor. The study results indicate that propranolol therapy, initiated early after MI, could save at least 6,500 lives each year in the U.S.

The BHAT findings generally comfirm those of a Norwegian study employing timolol, another beta blocker with properties much like propranolol, which also produced very promising results. The study involved 1,884 heart-attack survivors, again randomly assigned to drug-treated or placebo-treated groups and followed for periods up to 33 months (average followup: 17 months). Among drug-treated patients, timolol reduced overall mortality by 39%, recurrent heart attacks by nearly 30%, and sudden cardiac death (defined in this study as death occurring within 24 hours of new CHD symptoms) by 44%.

Treatment

Intensive coronary care units have been highly successful at reducing post-MI mortality from cardiac arrhythmias among hospitalized heart-attack patients, but have had only limited success in reducing deaths from acute heart failure and shock: conditions usually arising from extensive myocardial damage occurring during or early after the acute episode. Results from animal studies and very limited clinical experience have raised the possibility that substantial portions of heart muscle--threatened but not yet destroyed by blood deprivation (ischemia)--might be salvaged by interventions, begun early after onset of the attack, that would 1) improve perfusion of blood-deprived areas of myocardium, 2) facilitate the entry of nutrients and/or removal of potentially harmful metabolites, 3) reduce the cardiac workload and hence the oxygen needs of ischemic myocardium, or 4) counteract inflammation and cellular disruption in threatened areas.

The Multicenter Investigation for the Limitation of Infarct Size (MILIS) trial is evaluating two such interventions: hyaluronidase, an enzyme thought to facilitate nutrient entry and metabolite clearance in ischemic areas; and propranolol, a drug that reduces heart work and oxygen demand and prevents certain heart rhythm disturbances.

Enrollment of the 1200 heart-attack patients to participate in MILIS began in 1978, with participants being randomized either to a placebo-treated control group or to groups receiving hyaluronidase or propanolol therapy begun within 8-18 hours after appearance of MI symptoms. The goal is to ascertain whether either or both therapeutic regimens do, in fact, limit or reduce irreversible myocardial damage and, if so, to assess the

resultant effects on ventricular function and on subsequent morbidity and mortality. We are hopeful that these or similar approaches, if successful, not only will reduce mortality during the acute phase of MI, but also may reduce residual disability after recovery.

Another approach showing promise in the salvage of threatened heart muscle in evolving or acute heart attacks is the infusion of the fibinolytic agent streptokinase directly into coronary vessels that have become severely or wholly occluded by blood clots (thrombi). Thrombus formation on or in the vicinity of atherosclerotic deposits in coronary branches is the precipitating factor in most heart attacks (perhaps in over 70%). Streptokinase is an activator substance that catalyzes the conversion of plasminogen, an inactive blood protein, to plasmin -- a protein-digesting enzyme that can bring about the dissolution of blood clots by attacking fibin, an important structural component of the clot. In coronary thrombosis, intracoronary streptokinase infusions, if begun early after onset, may restore bloodflow to threatened heart muscle "downstream" from the thrombus and thus save heart muscle that might otherwise suffer irreversible damage from blood deprivation.

Though fibrinolytic therapy has been considered a significant advance over conventional anticoagulant therapy in the management of deep-vein thrombosis and potentially life-threatening episodes of pulmonary embolism, earlier studies evaluating fibrinolytic agents (streptokinase or urokinase) in the treatment of heart attacks had yielded unpersuasive results, and fibrinolytic therapy had sometimes been attended by serious bleeding episodes. In these studies, the drugs had been infused via a systemic vein.

More recently, investigators in this country and abroad have infused the fibrinolytic agent directly into clot-occluded coronary vessels via catheter, after first positioning the catheter tip as close as possible to the thrombus. This puts a high concentration of the fibrinolytic drug where it is needed. (However, other recent studies now suggest that effective intracoronary thrombolytic activity may be achieved also by intravenous administration of streptokinase at lower risk of complication.)

The perfusion time to clot lysis averaged about 30 minutes, with the occluded vessel being reopened 73-95% of the time. Although reocclusion subsequently occurred in up to a third of previously opened vessels, reinfusions of streptokinase sometimes succeeded in re-opening these vessels. The investigators feel that antiplatelet agents, such as aspirin and dipyridamole, may be useful in preventing re-occlusions, though this remains to be convincingly demonstrated.

In these studies, clot lysis was usually accompanied by significant relief of chest pain, though enzymatic and EKG evidence of infarction was often seen despite re-opening of the occluded vessel. Nevertheless, successful clot lysis apparently helped limit the extent of irreversible heart-muscle damage: in some studies higher ejection fractions were noted among streptokinase-treated patients than among controls, and in

another study thallium-201 scans were indicative of improved myocardial perfusion and heart-muscle salvage among streptokinase-treated patients.

Speed in initiating fibrinolytic therapy after onset of symptoms appears to be the major factor in myocardial salvage: best results were achieved when infusions were begun within 3 hours.

Although the results of the limited clinical experience to date appear promising, intracoronary infusions of streptokinase are not yet established therapy for coronary occlusion. However, a group of investigators have recommended that randomized, carefully controlled clinical trials be undertaken to establish the efficacy of this therapeutic procedure in heart-muscle salvage and to define its role in the treatment of acute heart attacks.

A Consensus-Development Conference on coronary artery bypass surgery, held at NIH December 3-5, 1980, concluded that, compared with conventional medical management, the operation improves life expectancy among patients with critical stenosis (greater than 50% narrowing) of the left main coronary artery. Surgery probably also improves longevity among patients with significant disease affecting all three major coronary vessels, though further definitive studies were thought desirable.

However, there is no evidence that surgery improves longevity in patients with single-vessel disease (other than in the left main coronary artery), and the evidence is mixed with respect to two-vessel disease: some studies indicating that surgery does not affect survival, others suggesting that it may improve survival among patients with two-vessel disease and moderate impairment of left ventricular function.

The skill and experience of the surgical team are prime determinants of the outcome of coronary artery bypass operations, with some surgical teams reporting operative mortality rates less than 1% and others rates in excess of 5%. However, data collected from 6,630 patients enrolled in the Coronary Artery Surgery Study have revealed a number of other factors that importantly affect surgical risk:

-- Age and sex of the patient. Among patients over 60, operative risk was unaffected by whether the patient was male or female. However, among patients aged 50-59, perioperative mortality was 4.5% for females versus only 1.9% for males.

-- Severity of preoperative angina. Patients severely incapacitated by angina prior to surgery had a perioperative mortality of 4.3%, versus 1.3% for those without angina.

-- Degree of heart failure. Indicators used were 1) a history of heart failure, 2) presence of pulmonary rales, 3) use of digitalis, and 4) use of diuretics. Patients with all four of the indicators had a 10% perioperative mortality, versus 1.4% among patients with none of these.

-- Number of coronary vessels involved. Among patients with single vessel disease, perioperative mortality was 1.4%; for two-vessel disease, it was 2.1%; and for three-vessel disease, it was 2.8%. Among patients with disease of the left main coronary artery, the degree of stenosis, if 75% or greater, affected operative risk. Stenosis greater than 90% was associated with a three-to fourfold increase in perioperative mortality.

-- Urgency of the surgery. Whether coronary bypass operations are considered elective, urgent, or emergency surgery is mainly determined by the patient's clinical status at the time of preoperative evaluation: the worse off the patient, the greater the urgency of operation in surgical candidates, but also the higher the operative risk. For example, in this study operative risk was 1.7% for elective surgery, 3.5% for urgent surgery, and 10.8% for emergency surgery.

All of the above are factors to be considered by the physician in weighing operative risk versus potential benefits in candidates for coronary bypass procedures.

The coronary Artery Surgery Study (CASS) is comparing medical versus surgical therapy for advanced coronary heart disease. The study involves 15 participating centers, a coordinating center, 780 volunteer patients, and a CASS registry of 24,188 patients. All patients are being followed for five years. Followup will be completed in 1983. Among still controversial questions that the study is attempting to answer: 1) Under what circumstances does coronary artery bypass improve life expectancy? 2) Does it reduce heart-attack risk or the threat of recurrent attacks among those who have previously experienced them? and 3) How does the quality of life over extended periods of time for surgical patients compare with that of patients receiving only conventional medical therapy?

Mitral valve prolapse (also called Barlow's syndrome, "floppy mitral valve," and mid-systolic click syndrome) is probably the most common condition affecting the heart valves. Estimates of the percentage of Americans with MVP range from 6-17%. In most people who have it, the condition causes no noticeable symptoms and requires no treatment other than reassurance; for the relatively few with associated incompetence of the valve, antibiotic prophylaxis is recommended to protect against bacterial endocarditis whenever dental or surgical procedures are anticipated.

But other MVP victims may suffer from bouts of chest pain, arrhythmias, dyspnea, anxiety, and other distressing symptoms. The condition may also place them at increased risk from sudden cardiac death or stroke. Usually, such symptomatic patients can be managed successfully with the beta-blocking agent propranolol; however, a small percentage of MVP patients may experience progressive mitral valve dysfunction eventually requiring surgical repair or replacement.

A recent study among children with MVP indicates that the condition usually has a benign course--at least during childhood and adolescence. The 119 subjects (average age 10 years at time of diagnosis) were followed for an average of 7 years. During this period only two required anti-arrhythmic therapy (for atrial tachycardia), one developed bacterial endocarditis, and one suffered a stroke. There were no sudden deaths and in none did the MVP progress to mitral-valve incompetence.

The results of transplantation studies in primates, reported last year from Stanford, had suggested that combined transplantation of the heart and lungs might be clinically feasible. During the past year, the Stanford team has performed the procedure in four patients, three of whom were still alive at the time of this writing.

The combined procedure is potentially lifesaving in patients with advanced bilateral lung disease, with or without heart failure, or in patients with primary heart disease complicated by secondary pulmonary vascular problems. It had been tried three times before--all more than a decade ago--but the longest survival had been only 23 days. However, the recipients of such transplants today should have a much better chance for long-term survival, thanks largely to the addition of cyclosporin A to the armamentarium of immunosuppressive drugs.

Cyclosporin A appears to inhibit T-lymphocytes, which play the principal role in rejection episodes, while leaving intact other elements of the body's immunological defenses against infection. This is an advantage over drugs used previously, such as the steroids, which impair immunological functions more or less indiscriminately, and lead to serious complications in many patients.

It is still much too early to predict whether combined heart and lung transplantation will enjoy anywhere near the same degree of success as has the Stanford heart transplantation program (currently about 70% are surviving at one year and 50% at five years). The Stanford team anticipates doing two to four of the combined procedures a year in the future.

LUNG DISEASES

The Institute conducts and supports research on the structure and function of the lung and on various diseases that afflict it. These include pediatric pulmonary disorders, emphysema and chronic bronchitis, fibrotic and immunologic lung diseases, pulmonary vascular disorders, and acute respiratory failure.

Respiratory distress syndrome (RDS) causes some 8,000-10,000 neonatal deaths each year in the U.S. Particularly vulnerable to RDS is the premature infant, whose lungs may not yet have matured sufficiently to meet its oxygen needs during the neonatal period. The Collaborative Study on Antenatal Steroid Therapy was prompted by findings from studies

in animals and some clinical results suggesting that steroids, administered to the mother late in pregnancy, could accelerate fetal lung maturation and thus protect against RDS.

The Collaborative Study results indicated that pretreatment of mothers with dexamethasone reduced the incidence of RDS among their premature female infants from one case in five to only one case in twenty, though it did not affect mortality among those infants developing RDS. However, the drug afforded no protection against RDS to premature male infants or to male or female infants of multiple births. Moreover, among female infants, the protection was not as great for whites as for other racial/ethnic groups. Despite these limitations, it is estimated that dexamethasone therapy has potential for preventing as many as 15,000 cases of RDS each year in the U.S. and thus for saving upwards of $200 million annually in costs of neonatal intensive care.

So far, the drug has not been found to have any short-term ill effects on the fetus, the newborn, or the mother. But all infants of dexamethasone-treated mothers will be followed for three years to ascertain whether there are any long-term ill effects.

NHLBI scientists have employed bronchoalveolar lavage to obtain information on inflammatory and immune effector cells and on non-cellular components from human lung in various disease states. Using a fiberoptic bronchoscope, they instill a small quantity of fluid to "wash out" some selected portion of the lower respiratory tract, after which some of the fluid, containing cells and proteins, is recovered for study. The procedure entails very little risk and causes little discomfort to the patient.

A number of lung diseases are characterized by the appearance in lung of large numbers of neutrophils. The NHLBI studies indicate that they are attracted to the alveoli from the pulmonary capillaries by a chemotactic substance which appears to be a lipoid compound of low molecular weight. The source of the chemotactic substance is the pulmonary macrophage, which releases it in response to various stimuli, including immune complexes, infectious organisms, or inhaled particulates, such as asbestos. Neutrophils contain a variety of substances capable of injuring lung tissues. These include oxidants and enzymes capable of digesting the collagen and elastin of lung connective tissue as well as other tissue components.

In addition to releasing the chemotactic substance, the pulmonary macrophage can produce a growth factor that stimulates replication of fibroblasts in lung. This factor is believed to play an important role in the lung scarring that occurs with interstitial lung diseases, such as idiopathic pulmonary fibrosis (IPF) and sarcoidosis.

Previously, it had been thought that increased net production of collagen by fibroblasts might be responsible for lung scarring in these diseases. But studies disclosed that fibroblasts from patients with IPF produced about the same amount of collagen per cell as did fibroblasts

from normal subjects. The discovery of the growth factor and its effects on various strains of human fibroblasts suggests that proliferation of fibroblasts in response to this substance may be a critical factor.

Other NHLBI studies suggest that a key role in IPF is also played by lung B lymphocytes. In the lungs (but not the blood) of subjects with IPF, B lymphocytes have been found that are capable of producing antibodies directed against components of lung connective tissue. The resulting immune complexes can contribute to the inflammatory process in the lungs and exacerbate the destructive processes.

In the alveolitis of sarcoidosis, the T lymphocyte appears to play a critical role. During active stages of the disease, increased proportions of T lymphocytes are found in the alveolar structures and there are shifts in the subpopulations of these cells--helper T cells being markedly increased and T suppressor cells decreased. Although this picture is seen at sites of disease activity, it is not reflected in blood samples drawn from sarcoidosis patients. As with neutrophils in IPF, the lung T cell population in sarcoidosis may be predictive of the subsequent clinical course. Patients in whom T lymphocytes make up more than 28% of the total lung population of immune and effector cells are likely to experience some deterioration in lung function over the next six months, whereas those with smaller proportions of T lymphocytes are likely to stabilize or improve over the same period.

In both IPF and sarcoidosis, therapy is directed toward suppression of the alveolar inflammation, usually with steroids. The NHLBI investigators found that low daily doses of steroids, supplemented by "pulse" treatment with single large doses (2 gm of methyl prednisolone) given by injection once a week, worked well in most patients with IPF. In patients with sarcoidosis, the same approach suppressed the alveolitis in 70% during the six-week period of treatment and this suppression persisted for at least six months. The investigators feel that the approach may circumvent some of the problems that often attend chronic steroid use while still adequately suppressing disease activity and preventing deterioration of lung function in sarcoidosis patients.

NHLBI was one of a number of organizations providing support for a Mount Everest expedition. In this case they were climbing the mountain in a quest for new knowledge about how the human body tolerates oxygen deprivation (hypoxia) under extreme conditions. Data on heart, respiratory, and various metabolic functions were collected at various altitudes between the teams's base camp at 17,700 ft and the summit (29,028 ft), which was reached by three scientists and two sherpas.

Measurements included electrocardiograms; arterial O_2 saturation; blood analyses for hemoglobin, lactate, and other components; and maximal work capacity and oxygen consumption. At the summit, using equipment modified to make it as light, small, and rugged as possible, the teams recorded air pressures and temperatures, their electrocardiograms, and their maximal ventilation while breathing ambient and/or oxygen-enriched air.

Mount Everest provided "laboratory conditions" that could not be duplicated by any existing research facility. It is expected that the ascent will provide data of value in research on various lung conditions characterized by hypoxia.

Patients with far advanced chronic obstructive pulmonary disease (COPD) often require supplementary oxygen, sometimes on a continuous basis. (A recently completed clinical trial among patients with end-stage COPD showed that oxygen therapy administered just at night was only half as effective in preventing death from COPD and its complications as was continuous oxygen therapy.) But continuous O_2 therapy by conventional techniques usually tethers the patient to his source of oxygen--whether it be a bulky tank, liquid oxygen container, or oxygen enrichment apparatus--and these permit him only limited mobility or none at all.

Recently, investigators have developed an oxygen delivery system that shows promise of changing this dismal prospect for some patients. Rather than delivering O_2 by a nasal cannula, which wastes a lot of oxygen, the new system delivers the gas through a thin catheter inserted through a "minitracheostomy" in the patient's throat, the catheter tip being positioned well down in the windpipe. So much more efficient is O_2 delivery by this technique that the patient's requirements can be met at O_2 flow rates as low as a tenth those required by conventional delivery procedures. The oxygen is supplied from a 6-lb. container, which the patient can carry in a shoulder harness and which can give him unrestricted mobility for periods up to 12 hours.

In animal studies and in very limited clinical testing in three patients, the system has posed no problems of infection, airway obstruction, or cartilage degeneration, and it has been well tolerated by the few patients who have received it. Although further testing is needed, the investigators feel that the system might benefit many victims disabled by COPD, fibrotic lung disease, or pneumoconioses.

Though lifesaving or life sustaining in many clinical situations, supplemental oxygen--especially in high concentrations for extended periods--poses the threat of injury to various species of lung cells. An excess of oxygen stimulates the production in these cells of superoxide anion, hydrogen peroxide, and hydroxyl radicals that may combine with the DNA or other components of the cell or else may cause peroxidation of lipids of the cell membrane. Vitamin E, certain enzymes (superoxide dismutase, catalase, and glutathione peroxidase), and intracellular reducing agents, such as ascorbic acid, glutathione, and cysteine, help protect the cell against oxygen toxicity. However, these defenses may be overwhelmed by persistent hyperoxia, resulting in cellular damage.

Even the pulmonary macrophage, a species of white cell that generates superoxides and other potentially harmful substances in the course of its battles against bacteria, particulates, and other "foreign" invaders, is not immune to effects of hyperoxia. Recent research indicates that

macrophages exposed to 40 and 60% oxygen for 48 hours exhibited some impairment in their ability to engulf bacteria as compared with macrophages exposed to room air (about 21% oxygen) for a similar period. Clinically, vitamin E and preparations of exogenous superoxide dismutase have been used with some success to counter oxygen toxicity. Nevertheless, although O_2 concentrations of 40% or less have not been shown to be harmful, it would appear prudent to employ the gas at the lowest concentrations needed for clinical effectiveness in patients requiring supplemental oxygen.

Another recent development that may help optimize respiratory therapy in patients with advanced lung disease is the percutaneous carbon dioxide sensor. It features a pair of electrodes that continuously monitor the partial pressure of CO_2 in the tissues. (which closely parallels that of arterial blood). Such information is particularly useful to the clinician in treating patients whose chronic lung condition is complicated by acute respiratory infections. Carbon dioxide retention is a frequent problem in such patients and supplemental oxygen is often needed.

However, unless the patient is on a mechanical ventilator, supplemental oxygen must be administered cautiously because the breathing of these patients may be more dependent than usual upon a hypoxic stimulus to the carotid or aortic bodies: chemoreceptors that respond to changes in blood oxygen or carbon dioxide levels by initiating signals to the brain centers that control respiration. Should blood oxygen levels be raised too high by supplemental oxygen, these chemoreceptors may be damped and the patient's breathing may be drastically slowed or may even cease. The percutaneous CO_2 sensor can provide much-needed information on blood gases in these patients while eliminating the need for repeated withdrawal of blood samples from the radial or femoral arteries--a procedure that not only causes discomfort to the patient but poses some risk (albeit slight) of hemorrhage or thrombosis.

A new technique of respiratory support, high frequency ventilation, shows promise of eliminating problems of overinflation and injury to lung airways that sometimes occur with conventional assisted-ventilation techniques. It employs extremely small volumes of gas delivered at high frequencies (usually 300-600 pulses per minute, though frequencies as high as 3000 pulses per minute are possible) via a small catheter positioned in the trachea. This provides adequate gas exchange in the lungs at airway pressures only a third those produced by conventional ventilatory techniques.

High frequency ventilation appears to hold promise for the treatment of respiratory distress syndrome and acute respiratory failure, though much additional research will be needed to establish its place in the clinical care of such patients.

The respiratory muscles of patients with chronic obstructive lung disease are under greater stress than those of normal subjects. Respiratory muscle fatigue can be an important factor limiting exercise tolerance in such patients, and it may also reduce their ability to cope with added respiratory burdens that may be imposed by acute respiratory illnesses.

However, recent research indicates that the respiratory muscles of COPD patients can be physically conditioned to increase their endurance and probably their strength as well. The inspiratory muscle training (IMT) exercises (inspiring against a resistance for two 15-minute periods each day) requires only simple, inexpensive equipment and the patient can follow the regimen at home without medical supervision.

In a recent study investigators compared the increase in exercise performance of COPD patients who followed the IMT regimen for two months with the performances of otherwise similar patients who participated in a regular hospital physiotherapy program (lifting small weights and exercising on a treadmill, cycle, and stairs three times a week). Compared with pretraining levels of exercise performance, the physiotherapy patients showed no significant improvement after two months. However, most IMT patients showed substantial improvement in the distance they could walk in 12 minutes and in endurance time while performing submaximal levels of exercise. Several IMT patients also reported that they could now perform routine household tasks with greater ease and suffered fewer episodes of breathlessness.

Endorphins are a class of peptides produced by the central nervous system and found in highest concentrations in brain and nerve tissues. Synthesized in increased quantities in response to pain, anxiety, and other stressful stimuli, endorphins are thought to modulate the body's reactions to these stressors. They appear to bind to the same sites in brain as do opiates.

In addition to relieving pain, opiates depress the brain respiratory center, thereby reducing respiratory drive and lung ventilation. For this reason, opiates are preferably not administered to patients with chronic obstructive lung diseases. Noting similarities between opiates and endorphins in site of action and in pain-relieving properties, some investigators have suspected that endorphins may also affect the respiratory center. In patients with COPD, the reasoning goes, chronic dyspnea could be a stressor causing increased synthesis of endorphins, which, in turn, might depress respiration and contribute to carbon dioxide retention in such patients. If so, then naloxone, an opiate anatagonist which works by competing with opiates for the same binding sites in brain, might also reduce the putative effects of endorphins on the respiratory center.

In one study, investigators report that naloxone did increase ventilation in some subjects with COPD, suggesting that 1) endorphins may interact with respiratory control mechanisms, possibly to reduce the intensity of the "stress" imposed by chronic airway obstruction, and 2) opiate antagonists might be useful in increasing respiratory drive and lung ventilation in conservatively managed COPD patients where CO_2 retention is a problem.

BLOOD DISEASES

Blood diseases addressed by NHLBI programs include sickle cell disease and other hemoglobinopathies, hemorrhagic diseases, and thromboembolic disorders. Other research is concerned with blood resource management and with improving the safety of blood and blood products.

Hemoglobin is assembled from two pairs of protein chains. During fetal life, two alpha chains are paired with two gamma chains to form fetal hemoglobin. However, shortly before birth a changeover occurs: production of gamma chains virtually ceases, production of beta chains begins, and thereafter alpha chains are paired with beta chains to produce adult hemoglobin.

In sickle cell disease, there is a single amino acid substitution (valine for glutamic acid) in the sixth position of the beta chain. The resulting molecules of sickle hemoglobin (HbS) tend to aggregate into a rigid gel under conditions, of low oxygen and distort the normally round red cell into a sickled shape. In Cooley's anemia the beta chain is normal, but is not made in sufficient quantities. This results in an excess of alpha chains that precipitate in the red cell, damaging it and shortening its lifespan.

Fetal hemoglobin transports oxygen as efficiently as normal adult hemoglobin does, but it does not have the beta chain that causes problems in sickle cell disease and Cooley's anemia. Thus, if fetal hemoglobin synthesis could be stimulated and maintained in patients with either disease, most of the associated clinical problems might be prevented or greatly ameliorated.

NHLBI scientists are studying hemoglobin switching in the sheep, which undergoes a changeover from fetal to adult hemoglobin synthesis that appears analogous to that occurring in man. In sheep, however, certain aspects of the changeover process can be manipulated reproducibly. In addition, recombinant DNA, molecular cloning, and cell transformation techniques are being employed in attempts to isolate alpha, beta, and gamma globin genes for study under defined conditions. By doing so, the scientists hope to learn how the gamma globin gene is "turned off" and the beta globin gene "turned on"--and vice versa.

An investigator at the University of Illinois has actually succeeded in stimulating fetal hemoglobin production in mature baboons by the drastic expedient of destroying a high percentage of the animals' red cells. This forced a high rate of production and rapid maturation of new red cells. Under these conditions the newly minted cells produced fetal hemoglobin in amounts up to 40% of their total hemoglobin content, but this high level of production could not be sustained.

Other studies have investigated whether inserting normal beta globin genes into bone marrow cells obtained from patients with sickle cell disease might induce these cells to produce normal adult hemoglobin in quantities sufficient to prevent sickling. In recent experiments, NHLBI scientists inserted copies of normal beta globin gene into cultured mouse fibroblasts. Though each cell injected received five copies of the gene, the inserted genes apparently were not stably integrated into the machinery of the cell nucleus and remained nearly dormant. Under conditions similar to those of this experiment, beta globin genes of normal bone marrow cells typically produce some 50,000 molecules of the messenger RNA that serves as the template for beta chain synthesis.

However, the transplanted genes managed only about 100, and it appears that none of these subsequently produced any beta chains. Moreover, the transplanted genes failed to respond to agents that normally activate slumbering genes.

More detailed knowledge of the molecular structure of sickle hemoglobin (HbS) and of the submolecular interactions that occur during its aggregation (first into long double strands and then into rigid cable-like fibers that distort the red cell into a characteristic crescent shape) have pointed up a number of chemotherapeutic approaches. Most are aimed at preventing HbS aggregation and polymerization in the first place, since once those cable-like fibers have formed, it seems unlikely that they can be readily unraveled by chemical means sufficiently mild to be tolerated well by the patient.

Research has shown that, when HbS gives up its oxygen, the HbS molecule undergoes a change in configuration. This apparently exposes sites on the surface of the molecule that can interact with similarly exposed sites on other deoxygenated HbS molecules in the vicinity and so initiate aggregation and polymerization. Oxygenated HbS is resistant to polymerization; hence another approach to the prevention of sickling has been to find reagents that will hold HbS in its oxygenated configuration even after it has given up its oxygen.

The key site for such a reagent to act appears to be a crevice separating adjacent beta chains of the HbS molecule, and thus called the beta cleft. This cleft widens when HbS gives up its oxygen and undergoes its conformational change. Thus the goal has been to find reagents that will fit into the beta cleft while the molecule is still oxygenated and which will react with groups on both adjacent beta chains to "bridge" the cleft. Presumably this would prevent widening when deoxygenation occurs and thus prevent the conformation change permitting interaction with other deoxygenated HbS molecules.

The most promising such agents to date are bis-salicylates (dubbed "two headed aspirin" by some investigators). Bis-salicylates have been synthesized that fit the beta cleft neatly and which will form the molecular bridges to "lock" the HbS molecule in its oxygenated conformation. Reacting these agents with bromine enables them to move readily through the red cell membrane, and the chemical products formed during the chemical bridging operation appear to be non-toxic. In the test tube, some of these agents have proved to be potent anti-sickling agents even in low concentrations. However, detailed animal testing will be needed to ascertain whether their action is highly specific for HbS or whether they may also interact indiscriminately with components of other cells, possibly with harmful results.

Patients with Cooley's anemia require repeated blood transfusions to keep their levels of red blood cells within tolerable limits. Each transfusion introduces substantial amounts of iron, which the body cannot

excrete readily and which it tolerates poorly. Eventually excess iron accumulates in various tissues and organs, including the heart. Cardiac complications of this iron overload, chiefly arrhythmias or congestive heart failure, are presently the leading causes of death among victims of Cooley's anemia.

To combat the iron overloading that occurs in Cooley's and other transfusion-dependent anemias, investigators have been seeking effective chelating agents, which combine with iron compounds to form complexes more readily excreted by the kidneys. One of the best iron chelators is desferrioxamine. However, its effectiveness has been limited by its rapid excretion after infusion, much of it being cleared before it has chelated any iron.

Earlier studies had demonstrated that slow subcutaneous administration of desferrioxamine by means of a small clockwork pump enhanced the iron-chelating effectiveness of the drug. More recently, investigators have found that incorporating the drug into red cell ghosts, which were then administered by slow intravenous infusion, made the drug still more effective. Though the drug-laden ghosts are themselves cleared fairly rapidly from the circulation, they persist longer than the free drug. Moreover, they are culled from the circulation by reticuloendothelial cells--themselves a major repository of excess iron--and so continue chelating to the very end.

Compared with subcutaneously administered desferrioxamine, the drug-laden ghosts more than doubled the patients' iron excretion. Also noteworthy were the high rates of iron excretion achieved among patients with relatively modest iron overloads. Such patients usually respond poorly to chelation therapy, and it is in such patients that the investigators feel the new technique will be most useful.

Enterobactin, a compound produced by the microbe E. coli, is the most powerful iron chelator known, but instability and other undesirable properties make it unsuitable as a candidate for clinical use. However, investigators have prepared sulfonated derivatives from several other compounds that were similar in chemical structure to enterobactin. In the test tube, these derivatives have been found to be chemically stable, are readily soluble in body fluids, and exhibit an exceptionally high affinity for iron--even removing it from the iron-transport protein transferrin, which desferrioxamine cannot do. They are presently undergoing testing in mice to ascertain their biological properties in vivo as well as any toxicity that they might exhibit.

A worrisome problem of blood or component replacement, particularly acute in patents with hemophilia or transfusion-dependent anemias, is the threat of transmitting hepatitis to these patients via virus-infected blood or blood products. With respect to hepatitis type B, this threat was substantially reduced by the development of tests that could reliably detect the presence of the virus in donor blood.

Subsequent research has resulted in the development of a hepatitis type B vaccine that has given excellent results in clinical trials among high risk subjects. The vaccine provoked an antibody response among 97% of those innoculated. The great majority mounted a strong antibody response that apparently conferred almost complete protection against hepatitis type B. There was also some evidence that the vaccine favorably modified the course of the disease when given to patients soon after infection with hepatitis B virus. The only side effect of the vaccine was temporary arm soreness in the vicinity of the injection site. It is anticipated that the vaccine will become generally available during the spring or summer of 1982, though limited supplies may restrict its early use primarily to high-risk individuals, such as transfusion recipients, hospital or dialysis center personnel, families of hepatitis carriers, and the like.

Now, with almost complete elimination of hepatitis type B-infected blood from the blood resource system, about 90% of cases of transfusion-transmitted hepatitis are of the non-A, non-B (NANB) variety, for which there is neither any vaccine nor specific blood tests to identify the infectious agent. However, recent studies have suggested that high levels of the enzyme alanine aminotransferase (ALT) in donor blood provide some basis for suspicion that NANB may also be present. These studies indicated that the risk of NANB among transfusion recipients was proportional to the ALT levels of the blood transfused, and was substantially increased if this blood contained more than 45 International Units of ALT per liter. The investigators calculated that excluding blood with ALT levels of 45 I.U./l or higher (these comprised about 3% of all units infused) might have prevented some 40% of the cases of NANB that developed.

Nevertheless, 63% of study patients receiving at least one unit of high-ALT blood did not develop NANB, whereas 19% of NANB cases occurred among patients who had received blood with ALT levels below 45 I.U./l. Thus high-ALT levels are not always indicative of NANB nor is all low-ALT blood necessarily free from the infectious agent.

Though some blood banks and medical centers have begun to screen donor blood for high ALT levels, such testing is not likely to be widely adopted unless and until more information becomes available on the causes of high ALT levels, their prevalence, and their persistence in the U.S. population. NHLBI plans to sponsor a special research effort to obtain this and other needed data.

In acute myologenous leukemia (AML) and certain other "stem cell leukemias," immature white blood cells are produced at an accelerated rate. They accumulate rapidly in bone marrow, where they impede production of red blood cells and platelets; and they often invade other tissues, particularly the liver, spleen, and lymph nodes. Most victims of these diseases are young children.

These forms of leukemia are usually fatal if left untreated, but remissions of varying duration may be achieved in most patients with intensive chemotherapy. Unfortunately, the massive destruction of white cells plus the associated bone marrow depression resulting from the chemotherapy reduce these patients' defenses against infections. An NHLBI-supported study investigated whether prophylactic transfusions of human granulocytes (a species of white blood cell) might provide some degree of protection against the infections that are prone to occur following chemotherapy.

Under the conditions of the study, they did not. The incidence of infections was about the same among patients receiving granulocyte transfusions as among controls (46% and 42%, respectively), although the transfusions did reduce the recipients' risk of septicemia. However, nearly three-fourths of the transfused patients experienced reactions (usually fever, shaking chills, and/or respiratory difficulties), and overall mortality was also somewhat higher among the transfusion recipients.

The only obvious benefit of granulocyte transfusion (some reduction in the risk from bacterial septicemia) appeared to be heavily outweighed by the substantial risk of transfusion reactions and other complications that may accompany such therapy. From the results of this study, the investigators conclude that prophylactic transfusions of granulocytes cannot be advocated as standard therapy during chemotherapy to induce remission in patients with AML. However, additional investigation has been called for by some scientists, who believe that larger quanties of granulocytes and more specific selection of donors could yield a different result.

DIV. OF HEART
& VASCULAR DIS.

DIVISION OF HEART AND VASCULAR DISEASES

ANNUAL REPORT

OCTOBER 1, 1980 - SEPTEMBER 30, 1981

General Mission

The mission of the Division of Heart and Vascular Diseases is to plan, direct, coordinate, and evaluate basic, applied, and clinical research, clinical trials, training and its development, and prevention, control, education, and demonstration in the field of heart and vascular diseases. The importance of this mission to the health of the country becomes clear when the magnitude of the problem is considered. Cardiovascular diseases rank first in causes of death and at least 40 million Americans have diseases of the heart and blood vessels. Even with the gradual decline in coronary and cerebrovascular disease mortality rates over the past 30 years, cardiovascular disease still accounts for over 50 percent of all deaths in the U.S. The burden of this enormous loss of lives in terms of productivity, economic costs, a personal loss, and suffering is virtually immeasurable. The Division of Heart and Vascular Diseases is responsible for planning and directing research activities that will result in a reduction in mortality and morbidity associated with cardiovascular diseases and eventually will lead to the primary prevention of these diseases.

Progress Toward Achieving Objectives

The major diseases addressed by the Division are arteriosclerosis, coronary heart disease, hypertension, cerebrovascular disease, cardiomyopathies, congenital heart disease, rheumatic heart disease and peripheral vascular diseases. Programs are implemented through research grants, specialized centers of research, direct staff research and management of contract-supported clinical trials or specialized research and development programs.

Basic Knowledge of Atherogenesis Continued to Increase Through DHVD Supported Research:

- A macrophage derived growth factor which stimulates _in vivo_ DNA synthesis and cell proliferation in vascular smooth muscle and endothelium has been identified. Since smooth muscle proliferation is considered to be a central event in the development of the atherosclerotic plaque. Further study of the mechanisms controlling production of the macrophage-derived growth factor may provide new insights into the pathogenesis of arteriosclerosis.

- A new *in vivo* assay of smooth muscle proliferation has been developed which allows study of aortic wall intima and media smooth muscle proliferation. The assay which has been carried out in rats and rabbits after stripping the endothelium of the aorta with an inserted balloon makes use of tritiated thymidine given as a bolus prior to sacrifice. Tritiated thymidine incorporation into DNA of smooth muscle has been found to be highly resproducible and predictive of intimal cell proliferation. This provides a new tool for studies of the influence of various agents on endothelial smooth muscle proliferation.

- The monoclonal hypothesis of origin of atherosclerotic lesions of the aorta has been given additional support from the study of fatty streaks considered precursors of atherosclerosis. Heptinstall at Johns Hopkins has found that aortal fatty streaks of black women at necropsy show intermediate clonal characteristics more frequently in middle age than at old age. This is interpreted that monoclonal fatty streaks go on to become fibrous plaques whereas polyclonal fatty streaks remain as fatty streaks. This finding could serve as an explanation for the previous observations that fatty streaks do not uniformly progress to more advanced atherosclerotic lesions.

- Prevention of hypercholesterolemia in cholesterol fed monkeys by alfalfa top saponins has been shown by Malinow as an extension of earlier observations of the anti-atherogenic and hypocholesterolemic properties of alfalfa meal. Feeding of this active component of alfalfa meal over an 8-month period resulted in plasma cholesterol values at 6 and 8 months of 161 ± 9 and 158 ± 9 mg/dl in the experimental group as compared with 342 ± 40 and 341 ± 36 mg/dl in the control animals. No signs of toxicity were seen as a result of this sapnonin feeding to reduce cholesterol concentrations.

- Extensive research continues in studies of lipoproteins, apoproteins and cell surface membrane receptors to seek better understanding of their roles in lipoprotein metabolism and in atherogenesis. Studies include the effects of diets and drugs affecting lipid metabolism in animals and in humans. Prevention of spontaneous atherosclerosis in the White Carneau pigeon has been demonstrated by dietary restriction limited to 40 percent of intake of controls beginning at 3 months of age. Regression of atherosclerosis in cynomolgus monkeys has been more difficult to achieve on low cholesterol, low fat diets than it is in rhesus monkeys which show rapid regression of both intracellular and extracellular lipid in the fibrous cap of lesions. Improved methods through immunoassays have been developed for all of the well characterized apoproteins making standardization between investigators is conducted in the SCOR's and in regular investigator-initiated projects.

- Coronary heart disease, the major clinical cause of morbidity and mortality from atherosclerosis, is being reduced in significant measurable ways. Between 1968 and 1978 there has been a reduction of 27.4 percent in coronary heart disease deaths. Despite an increase of 3 percent for deaths from all causes in 1980 related to the influenza epidemic and other reasons, there was still a 1.2 percent deadline in deaths from acute myocardial infarction. Interpretations differed as to whether these reductions reflect evidence of effects of reduction of risk factors for CHD, improved acute care or effects of postponed heart attacks among persons who have had coronary artery bypass surgery. It is likely that all are contributing to this reduction.

- CHD age-adjusted white male mortality trends by states, 1967-1977, revealed that West Coast states are decreasing the most rapidly and that Appalachian states are becoming those with the highest rates. This illustrates that the CHD mortality decline is not uniform throughout the U.S.

- Expanded knowledge of risk factors for coronary heart disease is resulting from LRC population studies, SCOR population studies and from direct epidemiological studies such as in Framingham and Honolulu. From the LRC program a monograph supplement to Circulation on the Epidemiology of Plasma High-Density Lipoprotein Levels provides population distributions of HDL cholesterol in relation to other coronary heart disease risk factors from childhood through adult ages. Risk factor profiles of children of parents with cardiovascular disease in a Louisiana Study clearly differed from the risk factor profiles of children of parents without cardiovascular disease. Framingham findings suggest that cholesterol levels change from 150 mg/dl in teen ages to adult levels of 200 mg/dl by mid-twenties to early thirties. The changes are largely associated with increases of body weight.

- The role of dietary factors in the genesis of coronary heart disease continues to stimulate debate. Data relating dietary nutrient intake to CHD and death in the Framingham, Honolulu, and Puerto Rico population studies showed a lower incidence of myocardial infarction and death from CHD among men who had greater caloric intake per kilogram of body weight; men who consumed more starch were less likely to develop MI or die from CHD; and men consuming more alcohol were less likely to develop CHD however they were more likely to die from other causes offsetting the favorable effects on CHD. Total fat or cholesterol intake was unrelated to incidence of CHD, however the percent of fat calories was higher among men who developed CHD. Dietary data on the 16,000 men followed for 6 years were reported in Circulation.

- Primary prevention of coronary heart disease through reduction of multiple risk factors is being tested in the MRFIT. This clinical trial conducted on 12,866 high risk men is 90 percent through its fifth year of followup and will terminate its clinical activities March 1, 1982. Long-term reduction of cigarette smoking and control of high blood pressure has proven possible through the preventive program. Reduction of cholesterol by diet has been less successful averaging 6.7 percent at the end of the fourth year. The morbidity and mortality experience will remain blinded until the results are reported in September 1982.

- Other clinical trials of primary or secondary prevention of coronary heart disease are in progress and will be providing evidence of the potential ability to reduce mortality or morbidity by drugs or by surgery. These trials are:

  <u>LRC Coronary Primary Prevention Trial (CPPT)</u>

  3,810 men and women randomized to the cholesterol lowering drug Cholestyramine or placebo control.
  Completion expected July 1983.

  <u>Beta-Blocker Heart Attack Trial (BHAT)</u>

  4,200 men and women with a documented myocardial infarction randomized to the Beta-blocking drug propranolol or to placebo control.
  Completion expected July 1982.

  <u>Multicenter Investigation of Limitation of Infarct Size (MILIS)</u>

  1,200 patients with acute myocardial infarction to be enrolled and randomized to either propranolol, hyaluronidase or placebo control.

  <u>Coronary Artery Surgery Study</u>

  780 patients with stable angina pectoris; postmyocardial infarction survivors without AP and other asymptomatic persons who have angiographic severe coronary artery disease have been enrolled into a 5-year followup study comparing medical versus coronary artery bypass surgery. Followup will end in 1983.

- The Albert Lasker Special Public Health Award for 1980 was presented to the NHLBI in recognition of the completion of the Hypertension Detection and Follow-up Program and its demonstration of a reduction in 5-year mortality among hypertensive men and women maintained under sustained blood pressure control by antihypertensive management. Further results from this

clinical trial involving 10,940 men and women from 14 communities have shown significant reductions of stroke morbidity and of development of left ventricular hypertrophy in the stepped care group, supportive of the mortality findings previously reported. Formulation of recommendations for practical management of high blood pressure for the 40 to 60 million persons who have this disorder will require careful judgments because of the costs and unknown longer term possible adverse effects of drug therapy.

- Renewed emphasis of research that may lead to understanding of pathogenesis of essential hypertension is recognized as a need. Directions being stimulated include approval of 10 new research grants ranking highest in merit responding to an RFA on "Studies on the Effects of Hypertension and Vasoactive Agents on the Vasculative". An earlier RFA on "Inter-discicplinary Studies on the Role of the Central Nervous System in Hypertension" has resulted in funding of 12 meritorious research applications. A new finding of evidence of renin-like substances widely distributed in human brain tissue has emerged, suggesting a more generalized function for brain renin than previously believed present. Other research in the roles of sodium, tyrosine and adenosine are adding further insights on mechanisms of blood pressure regulation.

- Combined heart and lung transplantation has been carried out as an experimental last resort for two severely incapacitated patients with end stage cardiac disease by Stanford surgeons. The use of the immunosuppressive drug, Cyclosporin A has made such surgery successful for these patients and its further use in cardiac transplantation should improve survival of transplant patients.

- Further progress with left ventricular assist devices is being made allowing temporary support of post operative patients recovering from reconstructive cardiac surgery. Implantable left heart devices have achieved over seven months of support function in a calf. Calcification of the device bladder is still an obstacle requiring solution.

- A new, highly promising defibrillator device has recently been developed. A report has been published describing the successful management of recurrent ventricular tachyarrhythmias in patients that were refractory to medical therapy.

Workshops, Task Forces

The DHVD sponsored 14 scientific meetings or workshops on research topics of emerging significance. Interchange of discussion among researchers accelerates advanced methodologies and brings more rapid evaluation of new technologies.

Percutaneous Transluminal Coronary Angioplasty Workshop (PTCA)

The rapid spread of use of angioplasty by balloon catheter to dilate coronary artery stenoses in patients with coronary artery disease had made evaluation of this procedure particularly timely. Clinical investigators participating in a voluntary registry of patients undergoing PTCA met in June to exchange experience with use of this procedure mainly in patients with single vessel disease. Successful dilatation is achieved in 60-70 percent of those considered suitable for the procedure. In-hospital mortality is 1 percent. Since coronary bypass surgery has now achieved less than 1 percent mortality inproficient hands, the further more formal evaluation of PTCA is needed to determine proper circumstances for its use.

Workshop on Cholesterol and Noncardiovascular Disease Mortality

The emerging question of a possible relationship of low cholesterol levels and increased risk of malignant neoplasms was the subject of a joint NHLBI-NCI workshop in May 1981. Investigators having major prospective population studies were invited to bring their data to analysis and such data from 17 studies were presented for evaluation by a review panel of scientific advisors. A significant inverse association of cholesterol levels with colon cancer in men has been documented in the Framingham, Honolulu, Hiroshima-Nagasaki and Stockholm Studies. The finding appears at levels below 190 mg/dl with a relative risk of about 1.7 when compared with the incidence among men 220-249 mg/dl. The association was considered more likely to be a secondary one to some underlying genetic or constitutional state than to a primary causal role.

The panel urged no let-up in efforts to get individuals with high cholesterol levels to reduce them to reduce risk of coronary disease. Men already at low levels would not be advised to attempt further reduction since the further reductions of coronary risk would be small and there might be an uncertain increase of risk of colon cancer risk at very low levels of cholesterol.

Major Problem Areas

- The personnel freeze on permanent category, experts and IPA candidates has been severely disrupting for program management and fulfillment of program responsibilities. The permanent personnel allocation for DHVD was reduced 10 positions from 129 to 119 in June 1981. Attraction of qualified professional staff and maintenance of high quality program management is made more difficult in this restrictive environment.

- Lack of growth of research funds is reducing the number of meritorious research projects which can be funded. The increase in the funds allocated to DHVD between FY 1979 and FY 1980 was 5 percent and between FY 1980 and FY 1981 was 3 percent. This occurred at a time when the cost of research was rapidly escalating.

  "Stabilization" of the number of new and competing investigator-initiated research grants supported by NIH was set at 5,000 annually. This coupled with resource limitations has resulted in a plateauing of total grants supported by DHVD at approximately 1,700 annually.

  Research training and development of new scientists is being impeded by uncertainties of authorizations and of funding levels. Shortage of investigators in specific areas has been identified and such shortages are unlikely to be corrected under the "stabilization" levels allowed to the DHVD. Particular categories of shortage of investigators are: clinical cardiovascular investigators, pediatric cardiology investigators, medical nutrition scientists with cardiovascular training, epidemiologists, biostatisticians, clinical investigators for clinical trials, cardiomyopathy research, peripheral vascular disease research and biomedical engineers.

DIVISION OF
LUNG DISEASES

DIVISION OF LUNG DISEASES

ANNUAL REPORT

October 1, 1980 through September 30, 1981

I. MISSION

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 disease (neonatal respiratory distress syndrome, cystic fibrosis, bronchiolitis); fibrotic and immunologic interstitial lung diseases; pulmonary vascular diseases (pulmonary hypertension, pulmonary edema); respiratory failure; and prevention, control and education. These programs are implemented through four branches: Structure and Function; Airways Diseases; Interstitial Lung Diseases; Prevention, Education and Manpower.

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

II. PROGRESS TOWARD OBJECTIVES

The investigator-initiated research grant continues to be the major source of innovative fundamental research. It contributes not only to our understanding of normal lung function and the abberant processes involved in respiratory disorders, but also leads to improvements in the diagnosis and management of pulmonary diseases. From time to time, the Division supplements these programs by issuing requests for applications (RFAs) or for contract proposals (RFPs) to encourage exploration of problems not being addressed, or being insufficiently addressed, by investigator-initiated grants. The areas where stimulation is needed are identified through working groups, workshops, and task forces, as well as by the Pulmonary Diseases Advisory Committee and the Division's professional staff. One program area in the Division's National Plan continues to depend almost entirely on special initiatives; namely, the Prevention and Control program.

A working group on the Natural History of the PiZZ Subject brought together investigators who had collected pulmonary function data on more than 300 subjects identified as homozygous for alpha-1-antitrypsin deficiency (PiZZ pheno-

type), a condition found in only about one in 1,500 individuals. At their first meeting, the data to be provided on individual subjects were discussed and a uniform format for their presentation agreed upon. Subsequent to this meeting, these retrospective data were submitted and analyzed. The findings will be discussed at a second meeting of this working group in the fall of 1981. This represents the largest currently available data base of changes in pulmonary function with time in PiZZ individuals.

The Division also sponsored two workshops to enable experts to discuss the current state of knowledge in the field and recommend new directions and goals to fill gaps in current research programs. Reports of these workshops, which are distributed to the biomedical community, serve as a stimulus for new investigative approaches, and provide a source of ideas for special initiatives of the Division of Lung Diseases. A workshop on Cardiopulmonary Resuscitation: The Role of Lung Mechanics reviewed and assessed the role of lung and chest-wall mechanics in sustaining peripheral blood flow through changes in intrathoracic pressure during cardiac arrest. Recent observations strongly suggest that the currently accepted explanation of how the circulation is sustained by sternal compression during cardiopulmonary resuscitation (CPR) does not apply in most instances. The recent work raises the possibility that blood leaves the thorax during compression because of the rise in pleural pressure. If this is true, it is likely that the current practical method of CPR is not optimal in sustaining circulation and that an alternative method of increasing pleural pressure may prove to be more efficacious. The Behavioral and Psychosocial Aspects of Respiratory Diseases was the subject of a workshop aimed at identifying existing behavioral knowledge and techniques which are applicable to the prevention of lung disease and the management of patients with lung disease and their families. The participants, representing the diverse backgrounds and expertise of physicians, pulmonary researchers, psychiatrists, psychologists, nurses, and educators, identified areas where additional research is needed and recommended specific approaches to address the behavioral and psychosocial problems associated with the prevention and management of pulmonary disease. The Division of Lung Diseases and the National Institute of Allergy and Infectious Diseases jointly sponsored a workshop on Basic and Clinical Aspects of Granulomatous Diseases which was held in June 1980. Participants included basic and clinical investigators with expertise in immunology, hypersensitivity, inflammation, granuloma induction, experimental models of granulomatous disease, and pulmonary disease. The report of this workshop has recently been completed and is being readied for distribution to the biomedical community.

The 11-member Task Force on Pulmonary Technology completed its deliberations in May 1981 and its final report will be distributed to the biomedical and industrial communities by September. The goal of the task force was to assist the Division in planning a responsive, well-focused program in pulmonary technology research, development, and evaluation. The final report contains specific recommendations for new and innovative technology research and development along with additional basic and clinical investigation upon which the development and application of new technology depends.

During the past year, the Nocturnal Oxygen Therapy Trial (NOTT) has been completed and the Intermittent Positive Pressure Breathing (IPPB) Trial has reached

its recruitment goal. The major outcome of the NOTT trial was published in the Annals of Internal Medicine and presented convincing evidence that in severe hypoxemic chronic obstructive lung disease, patients on nocturnal oxygen therapy have two times the mortality rate of those using continuous oxygen therapy. This finding, which has been widely disseminated, is expected to have a major impact on the treatment of patients with the disease. In the IPPB trial, approximately 1,000 patients have been recruited and baseline analyses completed. Some of this information has been presented at meetings of the American College of Chest Physicians and the American Thoracic Society and the initial papers are currently in preparation.

The collaborative, double-blind clinical trial to determine if antenatal administration of dexamethasone (a synthetic steroid) could reduce the incidence of neonatal respiratory distress syndrome (RDS) is still in progress. The infants born in the study are still being evaluated to determine the long-term effects of antenatal steroid therapy.

The Division of Lung Diseases is committed to periodic assessment of its special programs. Now that 14 awardees supported through the Pulmonary Academic Award Program have completed the 5-year term of this award, the Division issued an RFP to obtain an evaluation of this program. Respondents were asked to present proposals for comparing these 14 awardee schools, which had minimal pulmonary programs prior to the award, with a group of similar schools that had not received a Pulmonary Academic Award (PAA). The PAA evaluation contract, which was awarded to the Association of American Medical Colleges, is in its fourth-quarter and proceeding on schedule. Concurrently, information from centralized data sources such as the American College of Chest Physicians, the American Thoracic Society, and the National Board of Medical Examiners is being collected and analyzed to identify differences between study and comparison schools. The data collection efforts are nearing completion and a final report of the evaluation should be available in November 1981.

The NHLBI and NIAID have been awarded one percent set-aside funds to evaluate self-management programs and materials available to children with asthma, their families, and physicians who treat asthmatic children. The first phase of the evaluation project was a workshop, sponsored by NIAID, which was held in June 1981, at which the eight major research groups in asthma education presented their respective programs. The second phase of the project will evaluate these programs against specific criteria to determine the most effective elements of asthma education programs and recommend areas of needed research.

The Pulmonary SCOR (specialized centers of research) program was reopened to national competition for centers addressed to one of four disease categories: pediatric pulmonary diseases, chronic obstructive lung diseases, fibrotic and immunologic interstitial lung diseases, and pulmonary vascular diseases. Applications were due September 1980 and 21 out of the 24 submitted were subsequently approved by Council at its meeting in May 1981. At present, the Division expects to fund 16 SCORs beginning in fiscal 1982. SCORs addressed to respiratory failure were not included in this competition because their project period does not end until FY 1983.

As a special initiative to encourage studies in animals of the effects of nutrition on lung function, which has been little studied compared with the effects on other organ systems, the Division issued an RFA for studies of Nutritional Status and Nonrespiratory Lung Function. Twenty-six applications were approved (out of 45 submitted) by a special study section, and following Council approval, the Division initiated 6 awards in December 1980. These fundamental investigations are considered an essential step toward determining the role(s) of nutrition in the etiology, pathogenesis, and management of diseases of the respiratory system.

Another special initiative (RFA) invited grant applications on the role of Cell-to-Cell Interactions in the Developing Lung. Studies in organs other than lung have revealed that contacts between cells may involve short-range interactions between adjacent cells or interactions over long distances via chemical mediators. Information available on the lung in this regard is quite limited, and in particular, little is known about interactions between various cell types in the later stages of fetal and neonatal lung development. The fundamental studies supported by this RFA are expected to increase knowledge of the functions of individual lung cell types as well as the forms of communication between them. The Division hopes to initiate these awards in the fall of 1981.

In May 1981, the Division issued an RFP for a data and coordinating center to collect, analyze, and store data from approximately 35 clinical centers around the country that are participating in the Patient Registry for the Characterization of Primary Pulmonary Hypertension. It is estimated that a maximum of 200 patients, ranging in age from 1-year old to adults, who have pulmonary hypertension of unknown etiology, will voluntarily enroll in this registry per year over a 3-year period. The Data and Coordinating Center will develop a study protocol, collect the data submitted and monitor its quality, and perform the requisite laboratory and statistical analyses in accordance with the objectives of the registry. Applications for this RFP are due July 2 with the Division planning to make an award by September 1981.

Of considerable interest are new research emphases which are evident in several areas of investigation. These new avenues are the results of advances in computer technology and biomedical instrumentation, as well as new knowledge gained from clinical investigations, and information on the most basic structure and function of the lung. Some recent trends are identified below and specific examples given in the sections following.

The increasing interest in the research and development of sophisticated, often computer-based, biomedical instrumentation is giving rise to major new technologies which have the potential of becoming important tools for both biological research and diagnostic medicine. For example, qualitative, and in many cases, quantitative measurements of various respiratory and cardiopulmonary parameters can now be made using noninvasive methods. These include "on-line" and continuous measurements of arterial blood gas tensions, respiratory volumes and breathing patterns, regional distributions of ventilation and/or perfusion using computer tomography or radionuclide imaging, and measurements of pulmonary artery and, at least potentially, left heart pressures without cardiac catheterization.

The physiology and pathophysiology of the respiratory muscles during health and disease have recently attracted some attention. Recently, it has been demonstrated in healthy human volunteers that the respiratory muscles can be made to fatigue, giving rise to an inability to maintain stable arterial O_2 and CO_2 levels. Other studies have recently demonstrated that the strength and endurance of the respiratory muscles can be increased by training, in normal subjects, as well as in quadriplegics, patients with cystic fibrosis, and in patients with chronic obstructive lung disease. Thus, it is clear that the respiratory muscles can be trained for both strength and endurance, and regimens for maintaining training in the home are being developed.

The inflammatory process is critical to pulmonary fibrosis. Thus, various aspects of inflammation, including mediator substances and participating cells, are being actively investigated. It is anticipated that a thorough understanding of this process, including control mechanisms involved in the transition from acute to chronic inflammation, will facilitate therapeutic intervention and, thus, lead to effective management of patients in the early stages of pulmonary fibrosis.

Gas transport is classically approached in terms of cyclic ventilation where most of the transport of fresh gas is achieved by bulk flow through the airways, with mixing throughout the large alveolar compartment by diffusion. Recently, however, several investigators have demonstrated adequate gas exchange in animals and humans using small tidal volumes and at high oscillatory frequencies. By providing effective gas exchange at extremely small volumes, HFV has the advantage of requiring only negligible inflation (i.e., airway) pressures. Thus, problems of pulmonary barotrauma and overinflation would be greatly reduced or eliminated. Hence, HFV may have an impact on the care of newborn infants with RDS and in adults with chronic lung diseases such as emphysema and interstitial fibrosis. However, a great deal of basic research is still needed before this technique becomes a routine component of clinical care.

Asthma affects millions of children each year and continues to be the leading chronic disease causing school absenteeism. Asthma attacks disrupt family routines and prohibit many children from participating in daily activities. Yet, asthma attacks are often preventable if the patient takes responsibility for self-management of the asthmatic condition. An educational program recently developed has shown that children and their parents can often self-manage the condition without undue reliance on the medical system. The results of this project should be encouraging for community organizations, asthma clinics, and school systems, which are attempting to meet the educational needs of asthmatic children and their parents and will serve as a guide for future work in this important area.

The social and psychological consequences of chronic pulmonary disease for the patient and his or her family are becoming increasingly important as investigators select parameters by which to assess the efficacy of treatment regimens in various clinical trials. Greater emphasis on the "quality of life" emanating from various treatments represents a recent trend that is expected to continue. An educational program has recently demonstrated that

self-help workshops can teach COLD patients how to cope with problems arising from their disease and to become more active in managing their condition.

III. HIGHLIGHTS OF RECENT SCIENTIFIC ADVANCES

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

Recent studies have revealed that brain endorphins may be involved in the depression of ventilation which is seen in patients with chronic obstructive lung disease. Endorphins are a family of natural substances occurring in the brain which bind to the same receptors in the brain as opium-like drugs. A study of patients with chronic obstructive pulmonary diseases (COPD), who retain an excessive amount of carbon dioxide (CO_2), has shown that in some patients there is a definite increase in ventilation when an opiate inhibitor, naloxone, is administered. These studies have begun to shed light on mechanisms that might lead to CO_2 retention in COPD patients. For whatever reason, the brain's own natural opiate system appears to depress ventilation even though body CO_2 levels may be increased. While this may leave more "reserve" for increasing ventilation with exercise, the excess CO_2 does create problems, and in severe cases, can lead to coma and death.

Although pulmonary embolism is very prevalent and often occurs as a complication of chronic cardiac or pulmonary diseases, there is no reliable method currently available for diagnosing the disease. The most commonly used methods are ventilation-perfusion scans, a technique employing radionuclides for visualization of the ventilatory and circulatory compartments of the lung, and angiography, a radiologic technique that visualizes the pulmonary circulation. Both of these techniques have major inadequacies. For example, because ventilation-perfusion scans are often not specific enough, many patients who do not have pulmonary emboli are subjected to high-risk anticoagulant therapy. Angiography is more specific, but is expensive and requires specialized equipment and personnel. Thus, there still remains a great need for an accurate noninvasive test for pulmonary embolism.

A new technique which does show some promise has recently been applied to the evaluation of infants with respiratory distress syndrome. This new, noninvasive methodology, brainstem auditory evoked potential (BAEP), records the conduction of electrical potentials within the central nervous system, and may provide a sensitive index of its maturation and function. BAEP has recently been introduced as a new tool for evaluating infants with respiratory distress. Investigators tested two groups of infants (one consisting of full-term normal babies, the other of premature infants with respiratory distress) on several occasions from birth to 1 year of age and found a distinct difference between the two groups, particularly around 1 year of age. Whether this difference represents deficits in the central nervous system or simply a delay in development is not known and must await further test-

ing as the infants develop. However, the recognition of potential abnormalities this early in life is an important step towards better management of the increasing number of premature infants with respiratory distress whose life it is now possible to save, but who seem to be at risk for developing learning disabilities later in life.

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

In a study designed to identify childhood risk factors for the development of adult obstructive airways disease, respiratory histories and spirometric measurements were obtained on 650 children, 5 to 9 years of age. Persistent wheezing was the most frequently reported chronic symptom, occurring in 9.2 percent of these children. Interestingly, parental cigarette smoking was directly related to the occurrence of persistent wheezing in these children. Children who wheezed had histories of more respiratory illness (asthma, acute lower respiratory illness) than those who did not. When followed prospectively, the children who wheezed were seen to have higher incidences of acute illness of the lower respiratory tract. Whether these children will go on to develop airways disease as adults remains to be seen as the study unfolds. However, related studies from Australia which followed children reporting wheezing until they reached 21 years of age suggest that wheezing in childhood may be a risk factor for the development of obstructive airways disease in adulthood.

Hypersensitivity pneumonitis, as exemplified by farmer's lung disease, pigeon breeder's disease, and humidifier lung, to name but a few, is a complex immunologic lung disease which is being recognized with increasing frequency across the country. One of the great difficulties in studying this disease has been the lack of animal models which reflect the human condition. Such a model is now available which may hold the key to other fibrotic lung diseases as well. After repeated immunization with pigeon serum, monkeys are exposed to low-dose, whole-body X-irradiation followed by inhalation challenge with the antigen. The pulmonary disease which ensues is quite typical of the hypersensitivity pneumonitis seen in man. Not only is it expected that this model will be useful in studying the human disease, but it has, as part of development of the model, corroborated earlier suggestions that a defect in immune regulation may be important in disease production. In addition, it is hoped that this new model will contribute new information which will lead to better clinical care for diseased individuals.

Other recent studies have revealed that underlying pulmonary health may be an important factor in development of disease following exposure to silica dust. Experiments carried out in rats showed that rats with low-grade pulmonary infections are more likely to develop silicosis after exposure to silica than are respiratory disease-free rats. Thus, it appears that factors other than initial dust exposure may influence the eventual outcome of silica inhalation. These studies suggest that careful monitoring of the pulmonary health of individuals known to have been exposed to silica dust along with timely diagnosis and early treatment of incipient pulmonary infections may delay or even prevent the development of this debilitating diseases.

Other recent work underscores the importance of minimizing exposures of humans to high environmental and occupational levels of cadmium, a heavy metal found in air, food, water, and soil. It is inhaled with cigarette smoke and is an industrial pollutant which is released into the environment in massive quantities. The experiments demonstrated that cadmium severely alters the morphology of treated strips of tracheal epithelium. In addition, cadmium can cause significant reductions in ciliary motion, and can induce cell necrosis. These changes may explain the decreased mucociliary clearance and increased mortality from respiratory infections noted in cadmium-treated animals.

Other investigators are attempting to determine if exposure to moderate levels of oxygen is harmful. It has been well established that concentrations of 95 percent oxygen produce injury in a variety of experimental animals. Based on studies in normal subjects and patients, it has been generally accepted that oxygen concentrations of 40 percent or less do not produce significant lung injury. However, lung function measurements generally do not detect early injury and, presumably, large numbers of lung cells must be injured before such tests become abnormal. In this regard, a recent study found that exposure of isolated mouse lung macrophages in tissue culture to 40 and 60 percent oxygen for 48 hours resulted in significant depression of phagocytosis (the engulfing of foreign objects) as compared to air-exposed controls. Additional studies will be required to determine the biological and clinical relevance of these findings. However, the results of these studies suggest that lung cell injury may occur at lower levels of oxgyen exposure, and confirm the importance of using the lowest effective levels of supplemental oxygen in therapeutic regimens.

Investigations into how basic processes at the cellular level influence the etiology and treatment of pulmonary diseases continue to yield new and useful information. For example, the role of the extracellular matrix composed of collagen, elastic fibers, proteoglycans, and fibronectin in the development of both emphysema and adult respiratory distress syndrome (ARDS) has recently been recognized.

The extracellular matrix of the lung serves as the basic architectural framework of the organ. In emphysema, it is known that this framework is disrupted due to the degradation of elastin. This degradation apparently results from increased activity of the enzyme, elastase, caused by an imbalance in the normal levels of elastase and its inhibitors. Recent studies indicate that the disruption caused by elastase may extend beyond elastin degradation to the other components of the extracellular matrix, including certain types of collagen, proteoglycans, and fibronectin. In addition, it has recently been shown that interactions among matrix components may be important in determining the extent of matrix degradation. Pulmonary macrophages also appear to influence the degradation of elastin by acting on components of the extracellular matrix. Since it is known that more macrophages and elastase activity are found in the lungs of individuals who smoke and are at high risk of developing emphysema, extracellular matrix interactions will have to be considered as playing some role in the development of emphysema.

A new role for fibronectin in the development of pneumonia in ARDS patients has recently been postulated. Bronchopulmonary infection, particularly by

gram-negative bacilli, is a frequent and serious complication of human ARDS. These pneumonias reduce the chances of survival, prolong hospitalization, and possibly impair healing and recovery of the injured lung. The physical attachment and colonization by bacteria on the upper respiratory epithelium has long been recognized, but until now, the factors which actually mediate the bacterial adherence have been poorly understood. Recently, evidence has been found that the cells lining the respiratory tract are normally coated by the protein, fibronectin, which shield the attachment sites for gram-negative bacteria on the host cells. During the development of ARDS, proteolytic enzymes in the patient's respiratory secretions degrade the fibronectin, rendering the cell surfaces highly susceptible to bacterial infection. Once the identity of the proteolytic enzymes is established, it should open new avenues for the prevention of the fibronectin-depleting sequence which leads to bacterial colonization and pneumonia, and thereby improve the chances of successful recovery of the ARDS patient.

Injury to the pulmonary vascular endothelium is associated with a number of diseases involving the lung. The endothelial injury may be a primary event of the disease process. Mechanisms of endothelial injury are being studied using cultured endothelial cells from human autopsy material. Recent findings reveal that thymidine uptake (an index of cell growth and viability) is significantly depressed in cells exposed to sera from patients with adult respiratory distress syndrome (ARDS) and from patients with cardiogenic pulmonary edema. However, sera from patients with chronic obstructive pulmonary disease has no effect on thymidine uptake. Visual evaluation of the endothelial cells exposed to sera from patients with ARDS or cardiogenic pulmonary edema shows no evidence of cellular disruption or death. These studies demonstrate that serum factors in certain specific diseases are toxic for endothelial cells and that these factors may be important in the pathogenetic process.

The mechanism underlying the suceptibility of patients with cystic fibrosis to infections caused by *Pseudomonas aeruginosa* (PA) has also recently been investigated. Patients with cystic fibrosis frequently develop pulmonary infections caused by PA and this chronic infection is responsible for most of the morbidity and mortality associated with cystic fibrosis. These studies have revealed that although cellular and humoral immune mechanisms, in general, appear to be intact in cystic fibrosis patients, specific cellular immunity to PA seems to be impaired in patients with persistent PA infection. *In vitro* studies indicate that peripheral blood lymphocytes from patients with advanced disease fail to respond to PA and other gram negative bacteria. This selective lymphocyte dysfunction (unresponsiveness) in cystic fibrosis appears to correlate with progression of clinical disease. Related studies, suggest that the severity of the pulmonary disease in cystic fibrosis is related to the altered adherence and affinity for bacteria of certain cells. These studies suggest that susceptibility to *Pseudomonas* infection may be a characteristic acquired during the disease state. They also provide a new understanding of the cellular defects underlying the infections and possibly lead to rational approaches to the amelioration, or prevention of *Pseudomonas* infection in cystic fibrosis.

IV. REPORTS OF WORKSHOPS, MEETINGS AND OTHER ACTIVITIES

A. Workshop Reports

<u>Summary Report of Workshop on Noninvasive Methods to Monitor Intracellular Events</u>
NIH Publication No. 81-2108

<u>Report of the Workshop on Basic and Clinical Aspects of Granulomatous Diseases</u>
NIH Publication No. 81-2111

B. Evaluative Reports

<u>Evaluative Report on completed Contracts Awarded in Response to RFP NHLBI 75-1: Development and Clinical Evaluation of Blood Gas Sensors for Continuous Monitoring (Adult and Neonate)</u>
NIH Publication No. 81-2207

C. Other Reports

<u>Conference on the Scientific Basis of In-Hospital Respiratory Therapy November 14-16, 1979</u>
Am. Rev. Resp. Dis. <u>122</u>: Part II, 1980, pp. 61.

<u>Report of Task Force on Epidemiology of Respiratory Diseases</u>
NIH Publication No. 80-2019

<u>National Heart, Lung and Blood Institute, Division of Lung Diseases Program Report: Fiscal 1980</u>
NIH Publication No. 81-2267

<u>Report of National Heart, Lung and Blood Advisory Council</u>
November 24-25, 1980 (for limited distribution)

<u>Report of Task Force on Pulmonary Technology</u>
NIH Publication No. 81-2110

V. MAJOR PROBLEM AREAS

A. <u>Funds for New Initiatives</u>.

Because the Division is committed to investigator-initiated research as its first priority, and because the number of research grant applications addressed to pulmonary diseases continues to increase, it is very difficult to assign funds for activities that are necessary to fill gaps in research areas that still need attention and that are important to national health.

B. Funds for Research on Prevention, Education and Control of Respiratory Diseases

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

DIV. OF BLOOD
DIS. & RESOURCES

ANNUAL REPORT

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

October 1, 1980 through September 30, 1981

The Division of Blood Diseases and Resources seeks to improve the prevention, diagnosis, and treatment of blood diseases and related disorders and to assure the appropriate, efficient, and safe use of an adequate supply of high-quality blood and blood products. Its programs deal with four major areas: (1) Thrombosis and Hemostasis, (2) Red Blood Cell Disorders, (3) Sickle Cell Disease, and (4) Blood Resources. Each program encompasses 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, development and application of new knowledge so as to reduce morbidity, mortality, and the costs of health care. To best use its scarce resources, the Division actively coordinates its efforts with those of other Federal and non-Federal agencies and organizations. In developing and supporting its programs, the Division of Blood Diseases and Resources seeks to use all appropriate support mechanisms including: investigator-initiated research projects; program project grants; goal-oriented centers such as Specialized Centers of Research (SCORs), and a National Research and Demonstration Center; targeted research and development contracts; and evaluation and technology transfer projects. The long-range objectives of each Division program and one highlight from each program area of fiscal year 1981 progress are summarized in the following paragraphs.

THROMBOSIS AND HEMOSTASIS

The long-range objective of this program is to develop new knowledge that can be applied to prevention, diagnosis, and treatment of thrombosis and hemorrhage. The program has three subprograms. (1) Thromboembolic Disorders: This subprogram covers both arterial and venous thrombosis. Although the problems posed by these two disorders differ in many ways, elucidation of hemostatic mechanisms, including the roles of soluble clotting factors and their inhibitors as well as platelets and their secreted products, is important to both. The ultimate goal is the reduction of mortality and morbidity from venous thrombosis and occlusive arterial thrombi. (2) Hemorrhagic Disorders: This subprogram supports studies involving all aspects of the inherited bleeding disorders and also encourages basic research involving acquired disorders of coagulation. The overall goal is to develop a better understanding of the genetic and pathologic mechanisms underlying hemophilia and other bleeding disorders so as to develop improved diagnostic techniques and more effective specific treatments. (3) Platelet Disorders: This subprogram sponsors studies involving platelet function in relation to platelet disorders. The goals are to develop new knowledge of functional roles of platelets in the human biological defense mechanisms and to apply this knowledge to the development of more effective therapies for individuals suffering from congenital and acquired platelet disorders.

Highlights

Agents that dissolve blood clots (fibrinolytic agents) are important for restoring circulation through blocked blood vessels and for preventing damage to valves in veins. A new fibrinolytic enzyme, hementin, has been isolated. Hementin is capable of the rapid dissolution of blood clots. Because of this capability, hementin may prove to be a useful agent for the treatment of thrombotic disease.

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 and aplastic, hemolytic, and refractory anemias. It is composed of three subprograms. (1) Cooley's Anemia and Other Hemoglobin Disorders: The long-range goals are to improve patient treatment and extend the life span of afflicted individuals, as well as to improve the quality of life of victims of these disorders; to further elucidate basic defects, both genetic and biochemical, as a basis for developing techniques that can cure or prevent disease; to develop an understanding of the basic pathogenic mechanisms underlying the clinical aspects of the hemoglobinopathies; and to expand our understanding of the mechanisms of control of oxygen transport. (2) Erythropoiesis: The long-range objectives are 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; and to obtain adequate supplies of erythropoietin for research and clinical use. (3) Red Blood Cell Membrane and Enzyme Systems: The long-range goals are to further elucidate red cell membrane structure and function as well as gain additional insight into the process of intracellular metabolism; and to use this information to improve the health of patients afflicted with various hemolytic anemias.

Highlights

The treatment of certain genetic diseases, such as Cooley's anemia, requires chronic transfusion therapy. This produces a secondary hemochromatosis that causes buildup of iron in the tissues, eventually resulting in the failure of vital organs and in the death of most patients by early adulthood. Because there are limited physiological mechanisms in man for the removal of iron, the treatment of iron overload must rely on chelating agents that can affect iron excretion.

The siderophore (microbial iron-transport agent), enterobactin, is the most powerful iron-chelating agent found to date. While enterobactin itself is not a suitable candidate as an iron-chelating drug, similar substances are good candidates.

Investigators have prepared sulfonated derivatives of these chelators that combine several properties needed for an effective iron-removal agent. Like enterobactin, they have an exceptionally high affinity for iron, much higher than transferrin or Desferal. Unlike enterobactin, they are stable. In addition, sulfonation greatly increases the water solubility of these ligands

Recent studies have shown that, while the hydroxamate ligands such as deferoxamine (Desferal) are kinetically unable by themselves to remove iron from transferrin, these new ligands readily remove the iron from this iron-transport protein of human serum. While toxicity studies with mice for related compounds support the expectation of low toxicity for these compounds, their biological properties are being further determined by in vivo tests.

SICKLE CELL DISEASE

The mission of this program is to reduce morbidity and mortality caused by sickle cell disease. The long-range objectives are to continue basic research into the pathophysiology of the disease process at the molecular, cellular, and clinical levels; to develop improved methods of clinical care; to develop a more rational approach to patient management based on the latest scientific advances; to provide accurate, up-to-date information to health care providers and consumers; and to evaluate the effectiveness of education, screening, and counseling programs.

Highlights

Preliminary studies indicate that a direct analysis for the sickle cell gene mutation is now possible. Two restriction endonuclease enzymes have been found that recognize in normal hemoglobin the base sequence in the region in which a single substitution occurs in the sickle hemoglobin gene. Because of their specificity, they do not cleave the sickle hemoglobin gene in that region, and the resulting fragments have a larger molecular size than those from normal hemoglobin. Analysis of the fragments produced as a result of the action of these enzymes permits distinction between normal and sickle hemoglobin genes. Furthermore, the small amount of DNA required permits the use of amniocentesis rather than fetoscopy.

This new method for direct analysis of the gene mutation may provide an accurate, rapid, inexpensive, and safe means of diagnosing sickle cell anemia prenatally.

BLOOD RESOURCES

The blood resources program supports research activities to improve blood donor and recipient safety, blood component therapy, transplantation biology, blood substitutes, and all aspects of the management of blood resources including collection, fractionation, preservation, and distribution. This program consists of four subprograms with the following objectives. (1) Blood Resources Management: Foster the efficient use and assure an adequate supply of high-quality blood and blood products; promote more effective planning in the management of the national blood resource through collection and analysis of blood resource data; and encourage improved blood resource sharing both regionally and nationally. (2) Blood Safety: Promote basic investigations centering on immunohematologic problems particularly concerning blood group antigens and antibodies, the RH complex, and red cell antibody and complement interactions; promote studies to eliminate hepatitis as a transfusion-transmitted disease; and promote studies that will lead to greater safety for

donors of blood and blood components. (3) Blood Substitutes: Pursue further development of newly synthesized fluorocarbon compounds and other compounds for use as blood substitutes in transfusion therapy, organ perfusion, and other promising applications. (4) Blood Component Therapy: Support research on blood components including packed cells, albumin, granulocytes, and platelet concentrates; explore use of buffy coat as a source of human interferon; and develop new methods for plasma fractionation, including preparation of chemically useful trace components.

Highlights

Under the sponsorship of the NHLBI, the first major clinical trial of a new vaccine to prevent type B hepatitis has been completed. The vaccine consists of highly purified, formalin-inactivated virus subunits derived from the plasma of chronic carriers of type B hepatitis.

The vaccine was shown to provide virtually complete protection against the development of type B hepatitis. After booster shots, 96 percent of those innoculated showed increased antibody response rates through 18 months of followup.

The development of the hepatitis B vaccine and the demonstration that such a vaccine prevents HBV infection are major advances in the control of this extremely widespread disease.

Although most carriers of HB_sAg are asymptomatic, a substantial proportion eventually develop chronic active hepatitis and cirrhosis. There is also overwhelming evidence that the hepatitis B virus is the single most important causative factor of hepatocellular carcinoma. Thus, mass immunization programs against HBV infection may ultimately affect not only the incidence of acute hepatitis B and the pool of chronic carriers but may also reduce the morbidity and mortality from chronic active hepatitis, cirrhosis, and hepatocellular carcinoma.

DIV. OF EXTRA-
MURAL AFFAIRS

NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
DIVISION OF EXTRAMURAL AFFAIRS
Annual Report
October 1, 1980 - September 30, 1981

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

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

During FY 1981 the Division continued to provide a broad range of services for the entire Institute. These included:

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

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

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

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

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

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

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

8. Operation of the Program Policy and Procedures Office.

9. Committee management functions.

10. Training and orientation of extramural staff.

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

The initial scientific and technical merit review of special research grant applications, training grant applications and research and development contract proposals has continued to be the main focus of Review Branch activities. In FY 1981, the types of reviews in the grant program included:

Pulmonary Academic Awards
Preventive Cardiology Academic Awards
National Research Service Awards for Institutional Training Grants
Program Projects
Comprehensive Sickle Cell Center Supplemental Grants
Pulmonary SCOR Supplements
Specialized Centers of Research (SCOR): Pulmonary Diseases
Specialized Centers of Research in Arteriosclerosis (SCOR)
National Research and Demonstration Center Program (Supplement)
Clinical Trial Grants
Research Demonstration and Dissemination Project Grants
Conference Grants
Research Project Grants
Continuing Education Training Program
Clinical Investigator Awards (RFA)
Animal Models for the Study of the Pathogenesis of Specific Heart Muscle Diseases (RFA)
Cell-to-Cell Interaction in the Developing Lung (RFA)
Investigation of Basic Mechanisms Involved in Sudden Cardiac Death (RFA)

In FY 1981, numerous new, renewal, and unsolicited contract proposals were reviewed. In addition, the types of contract initiatives issued by the NHLBI for FY 1981 were:

Assessment of Ultrasonic B-Scan Imaging for Detection and Quantification of Atherosclerotic Lesions in Human Carotid and Iliofemoral Arteries and in Arteries of Animals (80-10)
Data Coordinating Center for the Assessment of Ultrasonic B-Scan Imaging for Detection and Quantification of Atherosclerotic Lesions in Human Carotid and Iliofemoral Arteries and in Arteries of Animals (80-18)

Research and Development of Percutaneous Energy Transmission System (81-1)
Coordinating Center for Community Surveillance (81-3)
Community Surveillance - Pilot Studies (81-5)
Primary Pulmonary Hypertension Registry (81-10)
Evaluation of Education Programs for Children with Asthma (81-11)
Support and Planning for Implementation of Smoking and Nutrition Education Program (81-12)
Pilot Evaluation of the SCOR Program (Resolicitation - See RFP 80-24)
Evaluation of Cardiac Function in Patients with Sickle Cell Disease: Special Study (81-15)
Coronary Drug Project - Continued Evaluation
Multicenter Investigation of the Limitation of Infact Size (Milis)

In the Division's Review Branch the Review Processing Section continued to perform its diverse functions in a satisfactory manner in spite of the large turnover in personnel. During this fiscal year the Processing Section changed leadership and replaced five grants clerks.

The Grants Operations Branch underwent a reorganization during the past year. The new Branch structure consists of the Office of the Branch Chief, three Grants Management Sections, the Awards Section, and the Council Services and Grants Records Section.

The Branch was involved in the Institute's grant activities from the pre-application (planning) stage to the close-out of terminated grant projects. Branch staff are often contacted by grantee institution staff concerning the best method of presenting consortium budgets, requests for research patient care costs, alterations and renovations, etc. The Branch was also actively involved in site visits, Council preparation, post Council - pre-award budget negotiations, issuing award notices (more than 3,600 totaling in excess of $372,000,000 for all the Institute programs) as well as the post-award fiscal and administrative management of those awards. The Branch served as an important interdivisional coordinator, and senior representatives of the Branch continued to be effective in assisting NIH-OD staff in the development and analysis of NIH grant policy and procedure. The Branch's continued efforts to improve management practices and procedures have undoubtedly resulted in considerable dollar savings for the Institute.

The Awards Section is responsible for the preparation of all award notices and grant encumbrance lists. The Section also receives, reviews, and files various documents pertaining to NHLBI grants and awards (e.g., activation notices, and Statement of Appointment forms). This Section is responsible for monitoring payback required of National Research Service Award trainees and for recording changes (budget period dates, change of P.I., etc.) for all active grants.

The Council Services and Grants Records Section continues to improve maintenance of grant records (pending, active and terminated). The Section files day-to-day correspondence and is responsible for daily update of the file charge-out system. This Section is also responsible for the

preparation of Council Books (the most recent Council involved more than 1400 summary statements) and for the duplication of some summary statements and supplemental material. Further, the Section is responsible for distribution of Council Books and preparation of the Council meeting rooms. The Section also provided information, duplicated from the official grant records, to the Institute's Privacy Act and Freedom of Information Coordinators.

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

During FY 1981, fifteen competitive contract solicitations (RFPs) were prepared and released by the NHLBI. As a result of these solicitations, it is expected that 27 new contract awards will be made. This will bring the number of active contracts for which the contracts staff is responsible to approximately 300, with a dollar value of slightly over seventy million.

There has been continued interest at the Departmental level in the even distribution of contract awards throughout the year, with particular concern for the avoidance of last-minute buying. In conjunction with this, a procurement planning schedule was developed at the outset of FY 1981 and updated on a quarterly basis. This schedule was maintained to assure an even distribution of awards and to guard against exceeding the maximum goal of 30% for fourth quarter awards established by the Department. Our latest estimate suggests that only 23% of our awards will be made in the fourth quarter. A preliminary schedule for FY 1982 has also been developed.

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

The contract operations of the NHLBI were reviewed by PHS procurement staff during December 1980 and January 1981. A draft report of findings has just been issued and that along with the exit interview conducted by the reviewers did not suggest that any truly serious irregularities were uncovered.

Employees of the COB were asked to complete a confidential questionnaire concerning a number of items related to working conditions in the Branch. The results suggested that the majority of employees are satisfied with their positions and the manner in which activities are managed. Some suggestions for changes in operating procedures were made and, where appropriate, these changes have been effected.

In an effort to assure that contract deliverables are received, a system was established whereby a monthly IBM System-6 printout, which outlines required reports for that particular month, is provided to each responsible Contracts Specialist. The Specialist then determines whether the report has been received and, if not, reminds the contractor of the delinquency and requests prompt submission of the report.

A Contract Format Handbook was developed for use by the contracts staff. This handbook provides staff with the lastest clauses and other pertinent information which must be included in contracts and assures considerable uniformity in the preparation of contract documents by various specialists in the Branch.

Procedures were changed so as to centralize most of the responsibilities for the closure of expired contracts in the DLD Contracts Section. This change relieves Contracts Specialists of much of the responsibility in this area, allowing them to devote more time to the negotiation, award, and post-administration of contracts. Through centralization, activities can be tracked on a daily basis and the needed emphasis given to this important program.

The Secretary has called for a review of the procurement management structure of the Department. The study is to be conducted with a view toward identifying possible areas of duplication, inefficient distribution of resources, weaknesses in management control or other unsound management practices. In anticipation of this review, the Director of the Division of Contracts and Grants, NIH, invited Mr. John Scully of the DHHS Office of Management and Budget to conduct a preliminary review of the NIH procurement function and to prepare a paper on his findings. A number of senior members of the contracts and program staffs had an opportunity to meet with Mr. Scully to discuss the NHLBI contract operations and to give their views on how well they are being carried out.

Mr. Curtis D. Tate, Chief, Contracts Operations Branch, was selected for the position of Deputy Director, Division of Contracts and Grants, OA, NIH. Mr. John A. Turlik, has been designated Chief with Mr. Robert R. Carlsen as Deputy Chief.

During the past year the Division has experienced a significant turnover of key personnel. The Division has been successful in filling these vacancies except one. In January 1980, Dr. W. Glen Moss retired as the Deputy Director of the Division. This essential SES position has remained vacant since his departure. A Search Committee has been established and a search plan formulated and approved; recruitment is now underway.

Overall, the Division has space to perform its various missions although the space in the Westwood Building is still spread over four floors and this causes considerable inconvenience. There are two specific areas where space needs are becoming very acute, however. In the Grants Operations Branch, the work-load relating to Council preparation has continued to grow so that the space allotted for that purpose is now quite cramped. In the Contracts Operations Branch, the accommodations for staff assigned to the Federal Building leave much to be desired.

DIV. OF INTRA-
MURAL RESEARCH

INTRAMURAL RESEARCH

THE NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

ANNUAL REPORT

October 1, 1980 - September 30, 1981

INTRAMURAL RESEARCH

Project Reports

Laboratory of Biochemistry

Summary-- 1
Metabolism of the branched-chain amino acids---------------------------- 19
Kinetics, regulation and mechanism of biochemical reactions------------- 23
Cellular regulation of enzyme levels------------------------------------ 31
Protein structure: Enzyme action and control---------------------------- 37
Role of selenium in anaerobic electron transport and in methane biosynthesis-- 50
Stereochemical studies of enzymatic reactions (5-deazaflavin)----------- 54
Mechanisms of cellular regulation - multiplemolecular forms of glutamine synthetase-- 58
Regulation of ammonia-assimilatory enzymes in Escherichia coli K12------ 61
Purine nucleosides as biological effectors------------------------------ 66
Enzyme mechanism and regulation--- 69
Inactivation of glutamine synthetase------------------------------------- 79
Selenoproteins: Synthesis and role of selenium-------------------------- 83
Purification of an enzyme of the first cycle in the covalent modification cascade of glutamine synthetase of Escherichia coli------------ 85
Development of a pure in vitro interconvertible enzyme cascade system--- 88
Menadione-dependent p-nitrophenyl-phosphatase of Clostridium sticklandii-- 90
Properties of 8-hydroxy-5-deazaflavin-dependent $NADP^+$ reductase from Methanococcus vannielii-- 93
Toxicity and transport of bilirubin-------------------------------------- 96
Regulation and mechanism of glutamine synthetase in E. coli------------- 99
Purification and characterization of selenium-containing transfer RNAs 103
Detection of cellular proteins containing tyrosine residues modified---- by phosphate or nucleotide phosphate---------------------------------- 106
Biosynthesis and properties of selenium-containing tRNA from Escherichia coli--- 109
Analysis of an inactivated form of the glutamine synthetase of E. coli-- 112
Oxidative inactivation of enzymes--------------------------------------- 115

Cardiology Branch

Summary-- 117
Natural history of asymptomatic or mildly symptomatic patients with coronary artery disease-- 127
The natural history of aortic regurgitation----------------------------- 129
Maintenance of a computerized clinical data bank for cardiology patients-- 132
Antiarrhythmic effects of verapamil in patients with hypertrophic cardiomyopathy-- 134
Verapamil: A new approch to the treatment of hypertrophic cardiomyopathy-- 136
Percutaneous transluminal coronary angioplasty-------------------------- 138
Specificity of systolic anterior motion of the anterior mitral leaflet-- 141
Distribution of hypertrophy by 2-dimensional echo in hypertrophic cardiomyopathy-- 143
Cellular disorganization in the left ventricular wall of patients with hypetrophic cardiomyopathy--- 145
NHLBI - Type II coronary intervention study----------------------------- 147

Ventricular septal contour in hypertrophic cardiomyopathy: Two-dimensional echo analysis-- 149
24-Hour ambulatory ECG monitoring as a predictor of sudden death in hypertrophic cardiomyopathy-- 151
Clinical utility of plasma verapamil levels in patients with hypertrophic cardiomyopathy-- 153
Clinical efficacy of verapamil alone and combined with propranolol in treating patients with chronic stable angina pectoris---------------- 155
Hypertrophic cardiomyopathy with unusual locations of left ventricular hypertrophy undetectable by M-mode echocardiography------------------ 157
Clinical and morphologic features of patients with hypertrophic cardiomyopathy and ventricular septal hypertrophy localized to the apical regions of the left ventricle ("apical hypertrophic cardiomyopathy")-- 159
Sudden death in hypertrophic cardiomyopathy: Clinical profile in 78 patients-- 161
Clinical features and natural history of hypertrophic cardiomyopathy in infants-- 163
Feline hypertrophic cardiomyopathy-- 165
Comparative effects of verapamil and nifedipine on exercise capacity and symptoms in patients with hypertrophic cardiomyopathy------------- 167
Regional reperfusion blood flow and extent of myocardial necrosis------- 169
Regional flow in ischemic myocardium: Effects of tachycardia----------- 171
Effects of verapamil and nifedipine on left ventricular function in HCM- 173
Left ventricular function during exercise in coronary artery disease subgroups-- 175
Improved left ventricular diastolic filling following coronary angioplasty-- 177
Body surface potential mapping in patients with hypertrophic cardiomyopathy-- 179
Prognostic importance of left ventricular ejection fraction response to exercise-- 181
Clinical efficacy of nifedipine with and without propranolol in patients with exertional angina pectoris due to coronary artery disease-------- 183
Comparative effects of verapamil, diltiazem and nifedipine on hemodynamics and left ventricular function-------------------------------- 185
Improvement of ischemia-induced delay in regional left ventricular emptying by verapamil-- 187
Functional mapping of regional time to minimum volume before and after coronary occlusion in the dog-- 189
Intramyocardial pH, drug, radionuclide and hemodynamic studies in myocardial ischemia-- 191
Experiments on pathogenesis of cardiac rupture and aneurysm formation following acute myocardial infarction-------------------------------- 193
Regional functional properties of the normal dog left ventricle as assessed by gated blood pool scintigraphy---------------------------- 195
The effects of reperfusion and retrograde bleeding on the results of programmed right ventricular stimulation------------------------------ 197
The natural history of electrical instability induced by programmed right ventricular stimulation after myocardial infarction in the dog-- 199
Regional left ventricular function after distal and proximal coronary occlusion measured by gated blood pool scintigraphy------------------ 201
Verapamil-induced changes in ventricular volume and diastolic function in hypertrophic cardiomyopathy-- 203
Scintillation probe measurement of left ventricular diastolic function in HCM-- 205

Hemodynamic effects of nifedipine in patients with hypertrophic cardiomyopathy---------- 207
Validation of intramyocardial fiberoptic pH probe for use in the study of myocardial ischemia---------- 209

Laboratory of Cell Biology
Summary---------- 213
Potentiometric studies of respiratory components of E. coli and rat liver---------- 219
DNA synthesis in E. coli---------- 222
Circular dichroic studies of protein denaturation---------- 224
Interaction of actin and myosin---------- 227
Respiration-linked H^+ ejection by reconstituted E. coli succinoxidase vesicles---------- 230
The binding of the subfragments of myosin to actin---------- 233
Models of muscle contraction---------- 237
Studies on plasma proteins in cystic fibrosis (CF)---------- 240
The mechanism of regulation of acto-S-1 ATPase by troponin-tropomyosin-- 243
Electrochemical potentials of protons in energy-transducing membranes--- 246
Structure-function relationships in eucaryotic cells---------- 249
Actin of nonmuscle cells---------- 252
Structure, assembly and function of microtubules---------- 257
Acanthamoeba myosins---------- 264

Laboratory of Cellular Metabolism
Summary---------- 269
Regulation of cyclic nucleotide phosphodiesterase activity---------- 277
Regulation of cAMP content of cultured cells---------- 279
Cyclic GMP metabolism---------- 282
Calmodulin-dependent cyclic nucleotide phosphodiesterase: Purification and characterization---------- 285
Production and characterization of monoclonal antibodies against bovine brain cGMP phosphodiesterase---------- 289
The mechanism and action of anti-inflammatory drugs; effects on neutrophil mobilization in carrageenan- and dextran-induced inflammation---------- 292
Role of histamine in gastric secretion. Studies with isolated gastric mucosal cells---------- 297
Regulation of histamine synthesis and release in tussues---------- 301
Reversible inhibition of cell proliferation in culture by anti-inflammatory drugs---------- 306
Regulation of cyclic nucleotide metabolism---------- 311
ADP-ribosyltransferases: Characterization of their substrates and factors that control their activity---------- 318
Interaction of GTP-binding proteins and catalytic component of adenylate cyclase---------- 321
Metabolism of lipids in human fibroblasts and muscle cells grown in culture---------- 324
Cellular distribution of histamine and its metabolizing enzymes in tissues: Studies with microvascular endothelial cells---------- 327
Regulation of hydroxymethyl CoA reductase in human skin fibroblasts----- 332
Effect of choleragen on guanine nucleotide release from turkey erythrocyte membranes---------- 335
Purification and characterization of a cyclis 3':5'-guanosine monophosphate-stimulated cyclic 3':5'-adenosine monophosphate phosphodiesterase from bovine liver---------- 338

Laboratory of Chemical Pharmacology

Summary-- 341
Kinetics of formation and elimination of drug metabolites by the liver 347
Regioselectivity in propranolol metabolism by rat liver microsomes------ 350
Guanylate cyclase: Preparation of monoclonal and polyclonal antibodies-- 352
Effect of chemical modification of prostacyclin on its action----------- 354
Role of calcium in CCl_4-induced hepatotoxicity------------------------- 357
Evidence by SEM for the mediator role of calcium in hepatotoxicity------ 360
Characterizatiuon of prostacyclin receptors in platelets---------------- 364
Inhibition of DNA synthesis by nitroso chloramphenicol: Protection by GSH-- 367
Studies on the renal reabsorption of cephalosporins in the rat---------- 339
Steady-state pharmacokinetics of p-aminohippurate (PAH) in rats--------- 372
Studies on the formation of reactive metabolites of phenacetin and acetaminophen-- 374
Interactions of doxorubicin with canine pulmonary microsomes------------ 376
Stereospecific dealkylation of oxyprenolol by mixed-function oxygenases-- 378
New metabolites of chloramphenicol-------------------------------------- 381
Induction of enflurane by hydrazine containing compounds---------------- 384
Mechanisms of formation of glutathione conjugates of bromobenzene------- 387
Stability of reactive intermediates of bromobenzene--------------------- 390
Electrophilic halogens as potential toxic metabolites of halocarbons---- 393
Effects of ketones on cytochrome P-450---------------------------------- 396
Development of a flow cell to detect cytochrome P-450------------------- 398
Cultured rat hepatocytes in the study of drug-induced hepatotoxicity---- 400

Laboratory of Chemistry

Summary-- 403
Nuclear magnetic resonance of natural products-------------------------- 409
Structure of natural products using instrumental methods---------------- 412
Characterization of natural products------------------------------------ 417
X-ray structural R & D for physiologically important molecules---------- 420
The characterization of natural materials------------------------------- 424
The use of digital computing in problems in biochemistry---------------- 427
Peptide biochemistry-- 429
Clinical biochemistry of the kallikrein-kinin system-------------------- 434
Biochemistry of the kallikrein-kininogen-kinin system------------------- 438
Hypotensive action of Hageman factor------------------------------------ 442

Clinical Hematology Branch

Summary-- 445
Molecular defect in beta thalassemia------------------------------------ 449
Cellular analysis of hemoglobin switching in sheep---------------------- 452
Regulation of the sheep globin genes------------------------------------ 455
Iron chelation in transfusional hemosiderosis--------------------------- 459
Hematopoiesis in bone marrow failure------------------------------------ 462
Transformation of mammalian cells--------------------------------------- 467
Use of hybridoma technology in the study of erythroid differentiation--- 470
Characterization of a repeated sequence and search for polymorphisms, in human DNA--- 474
Use of hybridoma technology in the study of erythroid differentiation--- 477
Characterizatin of the gene for human dihydrofolate reductase in normal cells and in cells resistant to methotrexate------------------------ 481

Laboratory of Experimental Atherosclerosis

Summary---------- 485
Localization of cholesterol in atherosclerotic lesions---------- 487
Quantification of circulating endothelial cells using flow cytometry---- 489
Cholesterol metabolism of cultured cells---------- 491

Hypertension-Endocrine Branch
Section on Experimental Therapeutics

Summary---------- 493
The search for renin-like activity in vessel wall---------- 499
The sympathetic nervous system and stress---------- 505
Baroreflex sensitivity in hypertension---------- 507
Effects of alterations of extra-cellular volume on urinary kallikrein excretion---------- 509
Collaborative studies of plasma catecholamines in man---------- 512
Impedance cardiography in hypertension---------- 514
The sympathetic nervous system and hypertension---------- 516
Studies of resistance vessels in hypertension---------- 521
Effect of aldosterone and vasopressin on kallikrein-like activity in the toad bladder---------- 523
Modification of endotoxic shock by vagotomy---------- 525
Role of vagal afferents in circulatory control---------- 528
Neuronal specific enolase in pheochromocytomas---------- 530
Naloxone reversal of Hageman Factor-Induced hypotension in the rat------ 533
Role of prostaglandins in primary aldosteronism---------- 535
Adrenergic nervous system function in experimentally-induced potassium depletion---------- 540
Effect of bradykinin on renal function in dogs treated with indomethacin or propranolol---------- 543

Section on Biochemical Pharmacology

Summary---------- 547
Mechanisms of storage, uptake and release of norepinephrine in adrenergic nerve endings (RT)---------- 555
Molecular biology to chemoreceptor regulation---------- 560
Functional role of calmodulin in the regulation of neurotransmitter receptors (Revised title)---------- 563
Biochemistry of the spontaneously hypertensive rat---------- 566
Regulation of tyrosine hydroxylase in the central nervous system-------- 569
Calcium-calmodulin dependent activation of tryptophan hydroxylase by ATP and magnesium---------- 572
The kinetic regulation of rat striatal tyrosine hydroxylase---------- 575
Adrenergic neurons in the brain---------- 578
Regulation of the Ca^{2+}-dependent regulator (calmodulin)---------- 580
Effects of pharmacological manipulations on the disposition of reduced biopterin (RT)---------- 583
Enkephalin-like peptides and cardiovascular control---------- 588
Development of chromatographic techniques for study of substance P in tissue samples. (Revised Title)---------- 591
Effects of morphine on calcium-regulated phosphorylation (Revised title)---------- 593
Characterization of a proenzyme I from rat brain (Revised title)-------- 596
Effects of pteridine cofactors on the stability of tryptophan hydroxylase and tryosine hydroxylase (RT)---------- 599
Calcium-regulated protein phosphorylation---------- 602

The role of the brain serotonergic neuronal system in blood pressure regulation---------- 605
Effect of cocaine on dopamine receptors and dopamine-sensitive cyclase (Revised title)---------- 608
Heat stable cytosolic factors afffecting synaptic membrane phosphorylation 610
The biosynthesis, distribution and biological role of substance P---------- 612
Cardiovascular DNA and RNA contents during development of cardiac enlargement in rats (Revised title)---------- 615
Serotonin receptors and cardiac ventricular sarcolemma---------- 617
Analysis of 6-phenylpterin as a cofactor for various hydroxylases from mammalian tissue---------- 620
Dopamine receptor regulation in schizophrenic illness---------- 622
Regulation and turnover of tetrahydrobiopterin---------- 624
Penetration of biopterin and other analogues into rat brain tissue---------- 627
Administration of tetrahydrobiopterin to Parkinson's patients---------- 629

Laboratory of Kidney and Electrolyte Metabolism

Summary---------- 631
Epithelial fluid transport and morphology---------- 637
Control of sodium and potassium transport in the nephron---------- 640
Hormonal control of transport in a line of toad kidney epithelial cells in culture---------- 643
Ionic composition of Necturus gallbladder cells---------- 646
Functional characteristics of the connecting tubule---------- 649
D-glucose transport across the renal proximal tubule brush border membrane 651
Study of transport by vesicles prepared from cultured epithelia---------- 654
Amiloride-sensitive volume regulation in Amphiuma red cells---------- 657
Culture and function study of epithelial kidney cells---------- 661
Urea transport in the kidney---------- 663
Isolation and study of brush border membrane transport proteins---------- 667
Acidification and bicarbonate transport by renal tubules---------- 670
Non-invasive studies of renal cellular energetics---------- 672
Solute and water pathways in epithelial barriers---------- 674
The sodium transport response to aldosterone-regulation by thyroid hormone 677
Prolactin control of ion transport and metabolism in cultured mammary epithelia---------- 680

Laboratory of Molecular Cardiology

Summary---------- 683
Smooth muscle and human platelet myosin light chain kinase---------- 685
Purification of myosin light chain phosphatases---------- 688
Studies with antibodies to myosin kinase isolated from smooth muscle---------- 690
Role of phosphorylation as a regulatory mechanism in muscle contraction--- 692

Molecular Disease Branch

Summary---------- 695
The biochemistry and metabolism of plasma lipoproteins---------- 705
Structure and function of plasma lipoproteins and apolipoproteins---------- 712
Molecular properties of lipoproteins and apolipoproteins---------- 717
Regulation of rat and human liver 3-hydroxy-3-methylglutaryl coenzyme A reductase by reversible phosphorylation---------- 731
Theoretical analysis of the metabolism of lipoproteins and their apolipoprotein and triglyceride moieties---------- 737
Metabolism of human plasma apolipoproteins---------- 744
Sterol metabolism in patients with betasitosterolemia---------- 747
Cellular lipid and lipoprotein biochemistry and metabolism---------- 749

Definition of the biochemical and physiological characteristics of hepatic lipase (HL) and lipoprotein abnormalities in primary biliary cirrhosis-- 752

Laboratory of Molecular Hematology

Summary-- 755
Molecular control of eukaryotic gene expression------------------------- 757
Regulation of protein biosynthesis in cell-free systems----------------- 760
Molecular cloning of eukaryotic globin gene sequences------------------- 765
Etiology of three mouse alpha-thalassemias------------------------------ 768
Correction of genetic defects by gene transfer-------------------------- 771
Chemical synthesis and molecular cloning of eukaryotic gene sequences---- 774
Regulation of eIF-2 activity: Model phosphoprotein and GTP-modulated regulatory protein-- 777

Office of the Director of Intramural Research

Section on Laboratory Animal Medicine and Surgery

Summary-- 781
Newfoundland breeding colony-- 783
NHLBI laboratory sheep colony--- 785
Indirect blood pressure measurements in laboratory animals using oscillometry-- 788

Section on Theoretical Biophysics

Summary-- 791
Computer simulation of renal function----------------------------------- 800
Theory of epithelial transport-- 803

Clinic of Surgery

Summary-- 805
Diastolic retroperfusion of acutely ischemic myocardium utilizing a balloon tipped coronary vein catheter------------------------------------ 811
Endothelial changes in human sapohenous veins prepared for coronary artery bypass grafts: Effects of distention pressure and preservation techniques--- 813
Morphometric analyses of myocardial hypertrophy (compensated and decompensated) utilizing computer-assisted microdensitometric techniques--- 815
Characterization of naturally occurring, genetically transmitted, fibrous subaortic stenosis in Newfoundland dogs------------------------ 817
Postoperative cardiac care in infants and children---------------------- 819
Evaluation of operative treatment for discrete subaortic stenosis-------- 821
Identification of human cardiac myosin isoenzymes----------------------- 823
Left ventricular hypertrophy produced in utero--------------------------- 825
Reperfusion with blood cardioplegia provides improved recovery of both early and late ventricular function after ischemic injury-------------- 827
Long-term results of repair of incomplete persistent atrioventricular canal-- 829
Quantification of regional myocardial dysfunction produced by reductions in coronary blood flow: Assessment by sonomicrometry techniques------- 831
Hemodynamic results following aortic valve replacement with the Hancock standard orifice bioprosthesis--- 833
Mitral valve replacement with a Hancock bioprosthesis - A five to ten year follow-up-- 835
Bioprosthetic valvular failure. Clinical and pathological characteristics in an experimental animal model------------------------------- 838

Microenbolization and left ventricular function during cardiopulmonary bypass---------- 840
Late results after operations for left ventricular outflow tract obstruction---------- 842
Influence of minoxidil on regional myocardial blood flow and cardiac pathology in beagle dogs---------- 844
Mitral valve replacement after closed mitral commissurotomy---------- 846

Laboratory of Technical Development

Summary---------- 849
Membrane lung systems for long term respiratory support---------- 857
Analysis of microcirculation by coherent light scattering---------- 866
Luminescence spectroscopy in biomedical research---------- 870
Methodology in fluorescence measurements research---------- 875
Blood flow measurements using nuclear magnetic resonance techniques----- 879
Instrumentation for the study of pre-steady state enzyme kinetics------- 883
Development of microcalorimeters and differential pH thermal titration apparatus for biochemical reaction studies---------- 887
Italy-U.S. Cooperative science program - blood gas instruments, project 78---------- 890
Development of electrochemical methods for kidney research and blood analysis---------- 893
Biophysical instrumentation for the study of protein dynamics---------- 897
Microcolorimetric measurement of magnesium ions---------- 901
New catheter idea to facilitate radiologic instrumentation---------- 904
New continuous extraction method with a coil planet centrifuge---------- 907
Preparative countercurrent chromatography with a rotating coil assembly- 912
Biomedical and biochemical applications of countercurrent chromatography---------- 917
Microfluorometer for analysis of femtomole amounts of biochemicals------ 922
Measurement of picomole amounts of ammonium ion---------- 924
Urinary valve prosthesis---------- 926

Pathology Branch

Summary---------- 929
Spontaneous exercise testing (running) as an early unmasker of underlying cardiac amyloidosis---------- 937
Examining the heart at necropsy---------- 939
Aortic dissection: anatomy, consequences, and causes---------- 941
Effect of chronic hypercalcemia on the heart. An analysis of 18 necropsy patients---------- 943
Prolapsing atrioventricuar valve in partial atrioventricular defect----- 945
Ebstein's anomaly of the tricuspid valve and prolapse of the mitral valve---------- 947
The case for preoperative coronary angiograpy in patients with tetralogy of Fallot and other complex congenital heart diseases------- 949
Hemochromatosis heart disease: an unemphasized cause of potentially reversible restrictive cardiomyopathy---------- 951
Mitral valve "anular" calcium forming a complete circle or "O" configuration: clinical and necropsy observations---------- 953
M-mode echocardiographic observations in active bacterial endocarditis limited to the aortic valve: an analysis of 17 patients---------- 955
M-mode echocardiographic observations during and after healing of active bacterial endocarditis limited to the mitral valve---------- 957
Postoperative aortic regurgitation from incomplete seating of tilting-disc occluders due to overhanging knots or long sutures---------- 959

Severe aortic regurgitation from systemic hypertension without aortic dissection) requiring aortic valve replacement. Analysis of 4 patients---------- 961
Comparison of amount and extent of coronary narrowing by atherosclerotic plaque and of myocardial scarring at necropsy in anterior-vs-posterior healed transmural myocardial infarction---------- 963
Type III hyperlipoproteinemia: quantification of amounts of coronary arterial narrowing in 5 necropsy patients---------- 965
Survival for 20 years or longer after transmural acute myocardial infarction: an analysis of 8 well-documented necropsy patients---------- 967
Fatal cardiac arrest during cardiac catheterization for angina pectoris: an analysis of 10 necropsy patients---------- 969
Amount of narrowing by atherosclerotic plaque in 44 nonbypassed and 52 bypassed major epicardial coronary arteries in 32 necropsy patients who died within 1 month of aortocoronary bypass grafting---------- 971
Ultrastructural evaluation of porcine valvular bioprostheses---------- 973
Pathology of saphenous vein aortocoronary bypass craft---------- 975
Structure of bovine pericardium and unimplanted pericardial bioprostheses---------- 977
Pathologic alterations in congestive cardiomyopathy of dogs---------- 979
Reduction of daunorubicin cardiotoxicity by ICRF 187 in rabbits---------- 981
Selenium-vitamin E deficiency in swine fed various metals---------- 983
Pathology of rheumatic heart disease---------- 985
Cardiotoxicity of adriamycin in rabbits---------- 987
Pathologic alterations in hypertrophic and congestive cardiomyopathy in cats---------- 989
Early calcification of a pericardial bioprosthetic valve---------- 991
Pulmonary langerhans cells in patients with fibrotic lung disorders---------- 993
Myocardial ultrastructure in the cardiomyopathies---------- 995

Laboratory of Biochemical Genetics
Summary---------- 997
Cell recognition and synapse formation---------- 1003
Acetylcholine receptors---------- 1009
Regulation of the biosynthesis and secretion of opioid peptides---------- 1012
The biology of cyclic nucleotides in *E. coli*---------- 1015
Metabolism of peptide hormones---------- 1017
Biochemical characterization of alpha-adrenergic receptors---------- 1019

Pulmonary Branch
Summary---------- 1023
Relationship of inflammatory and immune systems to the maintenance of lung structure and function---------- 1033
Clinical studies of lung disease---------- 1038
Control of synthesis and degradation of the extracellular matrix---------- 1049

Annual Report
Section on Enzymes
Laboratory of Biochemistry
National Heart, Lung, and Blood Institute
October 1, 1980 - September 30, 1981

A. Mechanisms of Enzyme Action

(a) Regulation of Enzyme Activities

(i) Studies with Permeabilized Cells. Previous studies have demonstrated that the state of adenylylation, $\bar{n}$, of glutamine synthetase (GS) in permeabilized cell preparations of *Escherichia coli* is determined by the concentrations in the suspending buffer of those metabolites that are known to affect *in vitro* activities of the bicyclic cascade enzymes that regulate the adenylylation and deadenylylation reactions. Of special significance are the findings: (1) that the value of $\bar{n}$ is a function of the ratio of glutamine to α-ketoglutarate (gln/α-KG) irrespective of the absolute concentrations of these effectors, and (2) that for cells in which only the adenylylation-deadenylylation cycle is operative (nitrogen-starved cells), a high gln/α-KG ratio is required to achieve a given value of $\bar{n}$ than is required for cells in which uridylylation-deuridylylation of the P_{II} regulatory protein is coupled to the adenylylation cycle. These results are in agreement with results obtained from computer simulations of theoretical steady-state functions derived for mono- and bicyclic cascade models. The results support further the conclusion that interconvertible enzyme cascades are not merely biological switches that turn "ON or OFF" biological functions, but are mechanisms by which the activities of enzymes can be geared to dynamic changes in the concentrations of regulatory metabolites.

(ii) Mixed-Function Oxidase Catalyzed Modification of Glutamine Synthetase. Previously (see last year's report), several different kinds of mixed-function oxidase systems from bacterial and mammalian sources, as well as a nonenzymic system comprised of ascorbic acid, O_2 and Fe(III), were shown to catalyze the inactivation of GS from *E. coli*. This inactivation reaction was inhibited by catalase and is modulated by the state of adenylylation of GS, and by the concentrations of ATP and glutamate. In the meantime, a physiological role of the inactivation reaction is indicated by the demonstration that inactivation of GS in nitrogen-starved cells is dependent upon the presence of O_2, glucose and Fe(III), is inhibited by Mn(II), dinitrophenol and arsenate, and is an inverse function of the intracellular level of catalase. With the assumption that glucose is essential for the generation of NADH and ATP, these properties are all properties of the *in vitro* enzyme catalyzed inactivation systems. Inactivation of GS by the ascorbate system and by a NADH-diaphorase-O_2 system involves the modification of just one of sixteen histidine residues in each subunit of GS. The modification leads to the formation of a new as yet unidentified amino acid derivative. Because all oxidase systems examined are capable of catalyzing the reduction of iron [$Fe(III) \xrightarrow{+e} Fe(II)$], it is proposed that this reduction may be involved in the site specific inactivation reaction. In this model, autooxidation of enzyme bound Fe(II) by O_2 could lead to an activated oxygen species (superoxide radical, singlet oxygen, hydroxyl radical, etc.) that could interact with a histidine residue at the active site and thus lead to inactivation. The NADH-diaphorase-O_2 inactivation system and the ascorbate system are being used to investigate the generality of this kind of enzyme modification reaction. Of considerable interest is the finding that rat liver glutamine synthetase is also inactivated by these systems. Among other enzymes tested, neither

β-galactosidase, β-glucuronidase, lysozyme from egg white, nor alkaline phosphatase from E. coli were inactivated by these mixed-function oxidase systems. However, β-galactosidase at one-tenth the concentration of GS subunits, protected the GS from inactivation by the diaphorase system.

(iii) Multiple Molecular Forms of Glutamine Synthetase. Studies on the separation of GS molecules containing different numbers of adenylylated subunits were continued. Irreproducibility of results obtained with affinity chromatography an Affi-blue sepharose columns was attributed to variations in the ambient temperature, and to the use of suboptimal levels of ADP and Mn(II) in the solution used for gradient elution. Atypical elution profiles which were obtained when stepwise gradients were used instead of continuous gradients are likely due in part to the presence of multiple forms of enzyme molecules containing the same number of adenylylated subunits (i.e., isomeric forms which differ in the distribution of adenylylated subunits), and also in part by inhomogeniety of the Cibacron blue-sepharose matrix with respect to affinity for adenylylated and unadenylylated subunits.

Scatchard plots of binding data obtained when GS is titrated with increasing amounts of anti-AMP antibodies are biphasic. From extrapolation of the linear segments of these plots, it is deduced that at low antibody/GS ratios, a single antibody molecule binds with high affinity ($K_d \approx 1 \times 10^{-7}$ M) to two different adenylylated subunits, either within the same GS molecule (monogamous reactions) or in different GS molecules (intermolecular cross-linkage), and at high antibody/GS ratios, each antibody molecule binds monovalently with low affinity (4.6×10^{-7} M) to a single adenylylated subunit. These results are in agreement with generally accepted theories for the interaction of bivalent antibodies with multivalent antigens to yield either precipitable aggregates at low antibody/antigen ratios or soluble complexes at high ratios. However, in this case, the failure to obtain quantitative precipitation of multiply adenylylated forms of GS (see last year's report) is likely due to variation in the distribution of adenylylated subunits that favor intermolecular cross-linking reactions on the one hand (to form precipitable complexes) and monogamous interactions on the other (to form soluble complexes).

(iv) Phosphorylation of Tyrosyl Groups of Proteins. The demonstration that phosphorylation of protein tyrosyl residues is associated with retrovirus induced cell transformation and the action of epidermal growth factor suggests that tyrosyl group phosphorylation may be important in the regulation of cell proliferation. A sensitive method for the detection and quantitation of phosphotyrosine in proteins has been developed. This method which involves rapid alkaline hydrolysis of proteins and quantitation of hydrolysis products by means of an amino acid analyzer is able to detect 100 to 500 pmol of phosphotyrosine (tyr-P). With this method, it was demonstrated that the egg phosphoproteins, phosvitin and vitellin, contain significant amounts of tyr-P, but no tyr-P could be detected in histone samples or in casein. These results invite speculation on a possible regulatory role of the egg phosphoprotein in cell proliferation (differentiation).

Antisera to tyr-P was produced by immunization of sheep with an albumin-tyr-P conjugate. The purified IgG fraction exhibited high affinity for tyr-P and low cross-reactivity with either phosphoserine, phosphothreonine, or tyrosine. The antibody protects phosphotyrosyl GS from digestion with alkaline phosphatase.

(v) Modulation Of Protein Phosphorylation by Purine Nucleosides. In view of the fact that purine nucleosides are known to serve as modulators of numerous

biological functions, the incidental discovery (see last year's report) that purine nucleosides stimulate the phosphorylation of proteins in porcine brain extracts is of considerable interest and potential significance. Further studies have shown that the nucleoside stimulation reaction occurs in brain, heart, kidney and testis, but not in liver. Calcium and magnesium stimulate the rate of phosphorylation. Calmodulin is probably not involved, since phenothiazine does not inhibit the effect of calcium. The possibility that the phosphorylation represents a step in the action of nucleoside phosphorylase is contraindicated by the fact that formycin B does not inhibit the phosphorylation reaction; moreover, addition of purified calf spleen nucleoside phosphorylase is without effect. In brain extracts, the nucleoside stimulated protein phosphorylation is accompanied by a reciprocal effect on the rate of ATP hydrolysis. This suggests the possibility that the role of nucleoside is to facilitate coupling of the generation of an unstable ATP derived intermediate with phosphorylation of the endogenous protein acceptor.

(b) Toxicity and Transport of Bilirubin

Hyperbilirubinemia is probably the most commonly diagnosed disease in newborn humans. Treatments are generally based on the theory that toxicity is due to passage through the blood-brain barrier of that fraction of bilirunin that is free (unbound). Alternatively, toxicity might be due to entry to the brain of albumin-bound bilirubin when under certain conditions the blood-brain barrier is opened. The latter possibility was confirmed experimentally by taking advantage of the fact that the blood-brain barrier can be opened reversibly by brief infusion of hypertonic solution into the carotid artery (Am. J. Physiol. 238, R421, 1980). Infusion of bilirubin peripherally into young adult rats in which the barrier was opened on only one side of the brain led to unilaterial kernicterus. Therefore, this approach provides a convenient animal model for further studies of kernicterus since a control hemisphere is available in the same animal.

Annual Report of the
Section on Intermediary Metabolism and Bioenergetics
National Heart, Lung, and Blood Institute
October 1, 1980 to September 30, 1981

Continuing research activities of the investigators in the Section on Metabolism and Bioenergetics are primarily concerned with (1) the isolation and characterization of bacterial selenoenzymes and seleno-tRNAs, the mode of biosynthesis of these selenium-containing macromolecules and their biological functions; (2) characterization of 8-hydroxy-5-deazaflavin dependent enzymes of Methanococcus vannielii and the electron transfer roles of these enzymes in formate metabolism and methane biosynthesis and (3) phosphate esterification and electron transfer reactions catalyzed by enzymes from Clostridium sticklandii. Together these processes constitute important aspects of the overall problem of the mechanism of electron transport and energy conservation in the low potential range of biological redox systems.

A. Bacterial Seleno enzymes and seleno-tRNAs

(1) Four bacterial enzymes that have been characterized as seleno enzymes in previous studies from this laboratory are clostridial glycine reductase, a formate dehydrogenase of Methanococcus vannielii, nicotinic acid hydroxylase of Clostridium barkeri and an acetoacetyl-CoA thiolase of Clostridium kluyveri. Recently another selenoprotein that exhibits hydrogenase activity has been discovered in M. vannielii and experiments are in progress to further characterize this new enzyme.

In earlier studies we could isolate reasonably stable derivatives of the selenium-containing moieties of glycine reductase Selenoprotein A and the selenium-dependent formate dehydrogenase and identify these as derivatives of selenocysteine residues present in the protein polypeptide chains. However, using similar procedures we have been unable to detect selenocysteine either in nicotinic acid hydroxylase or in thiolase. Instead, the only identifiable products isolated from these enzymes are dialkyl selenides which exhibit charge properties reflecting the charge properties of the alkylating reagents used for derivatization. For example, when ^{75}Se-nicotinic acid hydroxylase is reduced by its substrate, nicotinic acid, then allowed to react with an alkylating reagent and subsequently denatured by heat or urea treatment, the ^{75}Se is released from the protein in the form of a dialkyl selenide (alkyl-Se-alkyl). This indicates that the selenium present in the native reduced protein is in the form of an extremely labile selenol or seleno-keto compound or as a selenide complex. Preliminary studies are not consistent with selenium being present as iron-selenide (analogous to iron-sulfur centers of non-heme iron proteins). An alternative possibility that selenium might be present in the form of a molybdo-pterin cofactor is under investigation.

Initially it was thought that the selenium analog of pantotheine might be present in the seleno-thiolase and the actual Se chemical compound then would be selenocysteamine ($HSeCH_2CH_2NH_2$. However, the ^{75}Se-compound isolated from alkylated thiolase was easily distinguished from Se-alkyl-selenocysteamine and apparently was derived from some Se-cofactor that was decomposed during the derivatization procedure. In view of these results enzymic digests of the native ^{75}Se-labeled thiolase will be examined for the presence of the ^{75}Se-cofactor and also larger amounts of the enzyme will be examined spectrally in the ultra violet range in order to detect the Se-compound present.

(2) As one approach to addressing the problem of the mechanism of specific incorporation of selenocysteine residues in selenium dependent enzymes (e.g., clostridial glycine reductase selenoprotein A, bacterial formate dehydrogenase and mammalian glutathione peroxidase) we looked for the presence of a tRNA species that could be specifically charged with selenocysteine. Escherichia coli cells cultured under conditions that favor synthesis of the selenium-dependent formate dehydrogenase were used as experimental material. Earlier investigators had shown that under conditions of sulfur deprivation selenium amino acid analogs can substitute for the sulfur amino acids with the result that $tRNA^{cys}$ and $tRNA^{met}$ then are charged with selenocysteine and selenomethionine, respectively. However, under normal Se and S ratios neither of these selenoamino acids was found esterified to tRNA. Instead, tRNA species specifically modified with selenium in the polynucleotide portion of the molecules were synthesized. As previously reported for Clostridium sticklandii and Methanococcus vannielii, this incorporation of selenium into tRNAs by E. coli is highly specific and is not influenced by high levels of various sulfur compounds. The specificity of selenium incorporation was confirmed by two additional observations. Chromatographic separation of tRNA species synthesized in the presence of both $^{35}SO_4^{2-}$ and $^{75}SeO_4^{2-}$ revealed widely different patterns of labeling with the two isotopes. Furthermore, a mutant of E. coli unable to synthesize 4-thiouracil (the major thiobase of E. coli tRNA) synthesized normal levels of seleno-tRNA. In E. coli K-12 about one equivalent of Se is present per 70 mol of tRNA. In C. sticklandii and M. vannielii about 5 to 8% of the total tRNA populations contain Se-modified nucleotides. These organisms also contain conspicuous amounts of 4-thiouracil in their tRNAs. Nuclease digests of ^{75}Se-labeled tRNAs from both of these organisms contain a seleno nucleotide that is less acidic than 4-thiouridylic acid and on this basis is differentiated from 4-selenouridylic acid. Furthermore 4-Se-uridylic acid has an absorbancy maximum at 360 nm (compared to 335 nm for 4-S-U) but the unknown ^{75}Se-nucleotide exhibited no absorbance in this region.

Two highly purified seleno-tRNAs isolated from C. sticklandii by a combination of chromatographic techniques (reversed salt gradient, reversed phase and ion exchange) exhibit an unusual absorbance at 295 nm which may be a contribution of the selenobase present in the preparations. The charging activity of several preparations of one of these Se-tRNAs was investigated using both homologous and heterologous (E. coli) amino acid-tRNA synthetase preparations. None of the likely amino acid candidates (cys, glu, met or trp) was detected in ester linkage to the tRNA and thus its identity and/or role is still unknown.

Biosynthesis of the seleno-tRNAs has been shown to occur in bacterial cells rendered permeable to large molecular weight cofactors by treatment with detergents. Current studies are directed to obtaining a defined system in which the nature of the selenium donor can be determined. It is believed that such information also will be applicable to further studies on the formation of selenocysteine residues in selenoproteins.

As a means of facilitating isolation of seleno-tRNAs and seleno nucleotides or selenonucleosides for identification purposes, an affinity absorbent was prepared in which selenocysteamine was immobilized through a stable peptide linkage to CH-sepharose 4B. In the reduced form (-SeH form) this affinity adsorbent selectively retained ^{75}Se-tRNA which then could be removed from the column with a dilute borohydride solution. Glycine reductase selenoprotein A also was selectively adsorbed and could be eluted with a borohydride solution. In combination, this affinity material and organic mercurial affinity supports look promising for further purification of selenium-containing macromolecules.

B. 8-hydroxy-5-deazaflavin-dependent enzymes

A novel cofactor, 8-hydroxy-5-deazaflavin, is abundant in methane producing bacteria and this serves as electron carrier in coupled oxidation-reduction reactions wherein formate (via formate dehydrogenase) or molecular hydrogen (via hydrogenase) is electron donor. A reductase that specifically uses the deazaflavin as cofactor transfers reducing equivalents to $NADP^+$. The reaction mechanism involves a direct hydride transfer between the deazaflavin and $NADP^+$.

In recent studies a variety of analogues of the natural cofactor were synthetized and examined as substrates for the reductase. In this way the substrate structure-activity relationships could be assessed for the enzyme. The ability of the enzyme to reduce the various analogues using NADPH as electron donor was compared with the rates of reduction of the analogues with borohydride and cyanoborohydride.

Both the chemical and enzymic reductions gave rise to 1,5-dihydro-derivatives. Substitution of a CH_3 at C_5 for a H gave an enzymically inactive compound showing the importance of C_5 as the site of reduction. Since riboflavin, 1,5-dideazariboflavin and 2,4-dioxopyrimido[4,5-b]quinoline are enzymically inert the results suggest that the structural requirement of the chromophoric group as an enzyme substrate is 10-alkyl- or 8-hydroxy-2,4-dioxopyrimido[4,5-b]quinoline. Comparisons of effects of different substituents at C_3, C_7 and C_8 positions gave information concerning steric constraints at these positions for interaction with the protein.

Based on information from these studies an affinity chromatographic medium was designed for isolation of the reductase and other enzymes that utilize the 8-hydroxy deazaflavin as cofactor. Use of such an 8-hydroxy-deazaflavin affinity column allowed the isolation in essentially homogeneous form of a hydrogenase that copurified with ^{75}Se when ^{75}Se-labeled extracts were used as enzyme source. Additional preparations will be examined to ascertain whether this hydrogenase is indeed a selenoprotein. Like many other hydrogenases it is extremely oxygen sensitive and isolation procedures are carried out in the N_2 laboratory facility. A subunit molecular weight of 45,000 was estimated for the ^{75}Se-labeled protein under denaturing conditions.

Studies will be continued to detect other 8-hydroxy-5-deazaflavin dependent enzymes in *M. vannielii* using the affinity chromatographic approach.

Annual Report
Section on Protein Chemistry
Laboratory of Biochemistry
National Heart, Lung, and Blood Institute
October 1, 1980 to September 30, 1981

Research in the Section on Protein Chemistry consists of studies on the physical and chemical properties of macromolecules of biological interest and on the roles of ligand binding and of protein-protein and inter- and intra-subunit interactions in enzyme catalysis and regulation.

Glutamine synthetase, a strictly regulated enzyme in Escherichia coli, is a dodecamer with each subunit containing a catalytic site with two essential divalent cation sites (n_1 and n_2) and a tyrosyl residue which is the site of covalent modification by enzymatically-catalyzed adenylylation-deadenylylation reactions. Studies of the interactions of divalent cations, substrates, substrate analogs, and inhibitors with glutamine synthetase from E. coli have continued. Recent efforts have been directed towards characterizing the interactions of L-methionine-S,R-sulfoximine with glutamine synthetase since this active-site analog promotes conformational differences between the manganese, unadenylylated enzyme ($MnGS_{\bar{1}}$) and adenylylated enzyme ($MnGS_{\overline{12}}$). The resolved S- and R-diastereoisomers, as well as an equimolar mixture of S- and R-isomers, of L-methionine sulfoximine produce the same UV difference spectrum with $MnGS_{\bar{1}}$ or with $MnGS_{\overline{12}}$. These active-site analogs promote the burial of ~ 1 tyrosyl residue per unadenylylated subunit with $MnGS_{\bar{1}}$, whereas covalently bound adenylate groups are perturbed instead with $MnGS_{\overline{12}}$. UV difference spectra are due to local perturbations of aromatic residues produced by binding active-site ligands. A stoichiometry in binding of 1 eq of active-site ligand per subunit of dodecamer was confirmed in binding measurements with the S-isomer. Negative cooperativity in binding with Hill coefficients (n_H) < 1 is observed with the S-,R-, and the mixture of S- and R-isomers of the analog. Half-saturation values are ~3-fold greater with $MnGS_{\overline{12}}$ than with $MnGS_{\bar{1}}$. The relative affinity ratio for S:R isomers of L-methionine sulfoximine is ~ 10:1. However, with an equimolar mixture of S- and R-isomers of L-methionine sulfoximine at half-saturation of $MnGS_{\bar{1}}$, the binding of the S-isomer to some subunits of the dodecamer enhances the affinity of other subunits for the R-isomer, reducing the relative affinity ratio to ~ 3:1 for S:R isomer binding. The Mn·ADP·$GS_{\bar{1}}$ complex has a 200-fold greater affinity for the S-isomer of L-methionine sulfoximine than has $MnGS_{\bar{1}}$. Furthermore, the presence of ADP increases the affinity ratio for S:R isomer to ~ 340:1, indicating that the nucleotide changes the enzyme conformation at the active site in such a way that markedly favors the binding of the S-isomer over that of the R-isomer. Significantly, as Meister and coworkers have shown, it is only the S-isomer which can be phosphorylated by the enzyme in the presence of ATP and Mn^{2+} (or Mg^{2+}) to form the transition state analog L-methionine-S-sulfoximine phosphate. Synergistic effects are observed also between the binding of nucleotides and substrates (L-glutamate or L-glutamine) to glutamine synthetase.

Calorimetric studies with the resolved S- and R-isomers and an equimolar mixture of S- and R-isomers of L-methionine sulfoximine have been performed with manganese, unadenylylated glutamine synthetase ($MnGS_{\bar{1}}$) and the fully adenylylated, manganese enzyme ($MnGS_{\overline{12}}$). The intrinsic heats of binding the S-isomer and the R-isomer to $MnGS_{\bar{1}}$ appears to be about the same ($\Delta H \simeq -12$ kcal/mol of subunit), with a negligible proton effect associated with binding in either case. However, large differences in binding enthalpies and related proton effects are observed

in binding these active-analogs to $MnGS_{\bar{1}}$ and to $MnGS_{\overline{12}}$, suggesting that the conformations of $MnGS_{\bar{1}}$ and $MnGS_{\overline{12}}$ differ. The difference between conformations of the unadenylylated and adenylylated enzymes could be subtle, reflecting principally protein group pK perturbations produced by covalent modification. The observed proton effects on binding active-site analogs to $MnGS_{\bar{1}}$ and $MnGS_{\overline{12}}$ could relate to the difference in pH-activity profiles of these enzyme forms. Calorimetric studies with the equimolar mixture of S- and R-isomers suggest that 0.7 eq of H^+ uptake/subunit is associated with subunit interactions when the isomers are binding together to the dodecameric enzyme. Values of $\Delta G'$ from binding studies and ΔH values from calorimetric studies give $\Delta S \simeq -23$ kcal/(deg·mol of enzyme subunit) for the binding of the S-isomer or R-isomer of L-methionine sulfoximine to $MnGS_{\bar{1}}$ at pH 7.1 (30°C). This degree of overall ordering on binding the substrate analog is about the same as that obtained previously for L-glutamine binding to glutamine synthetase.

The auto-inactivation of glutamine synthetase in the presence of L-methionine-S-sulfoximine, ATP, and Mn^{2+} (or Mg^{2+}) is caused by the extremely tight (synergistic) binding of 2 Mn^{2+} and the phosphorylation products, L-methionine-S-sulfoximine phosphate and ADP, to each active site. At neutral pH, the inactive complex cannot be disrupted by EDTA and heat. It has been discovered, however, that after auto-inactivation, reactivation of the enzyme can be accomplished by incubation of the inactive enzyme complex at pH < 5 in the presence of high concentrations of salt. The rate of the reactivation process is first order (with a half-time of 2 minutes at pH 4.1 and 37°C in the presence of 1 M KCl) and corresponds to the rate of release per subunit of L-methionine-S-sulfoximine phosphate, ADP, and 2 Mn. The rate of reactivation is dependent on temperature and the 3rd or 4th power of the hydrogen ion activity. The proton dependence suggests that the protonation of 3-4 carboxylate groups per subunit is involved in the reactivation mechanism. It is likely that a protonation of carboxylate groups in Mn^{2+} binding clusters would sufficiently decrease the affinity for Mn^{2+} ions to allow dissociation of all components of the complex. From the temperature dependence of the reactivation process, an Arrhenius activation energy of 26 kcal/mol is estimated. The salt and temperature dependence of reactivation suggests that even at low pH, structural constraints exist within the enzyme which stabilizes the complex, possibly by making carboxylate groups less accessible for protonation. The inactive unadenylylated complex containing Mg^{2+} and the adenylylated, manganese enzyme complex are more rapidly reactivated than the unadenylylated, manganese complex under comparable conditions; nevertheless, the pH dependence and the Arrhenius activation energy are the same for reactivation of the inactive, manganese and magnesium enzyme complexes. The relative stabilities of inactive glutamine synthetase complexes are consistent with the relative affinities of the different enzyme forms for ligands.

After auto-inactivation of glutamine synthetase and reactivation of the inactive enzyme complex at pH < 5, neutralization of the solution was found to cause reinactivation of the enzyme. The addition of 1 mM EDTA completely blocks reinactivation and the addition of 1 mM Mn^{2+} accelerates reinactivation. Thus, reinactivation produced by the binding of L-methionine-S-sulfoximine-P and ADP requires Me^{2+} ions. With added 1 mM $MnCl_2$ at pH 7.2, almost total reinactivation of the enzyme was observed within 6 minutes at 25°C with enzyme concentrations as low as 0.2 μM subunits. At pH 7.2 with all dissociated components from the reactivation present, the affinity of the enzyme for any one component is estimated to be > 10^9 M^{-1}. However, the addition of excess ADP-Mn to the reinactivation reaction was quite inhibitory, suggesting that the nucleotide has a dual role in the binding process.

In stability studies of the inactive enzyme complex containing Me^{2+}, L-methionine-S-sulfoximine-P and ADP, information was obtained on the effects of bound active-site ligands on the tertiary and quaternary structure of dodecameric glutamine synthetase. Whereas the native protein is dissociated and unfolded rapidly in 6 M guanidine·HCl at 37°C, it takes 3-4 hours to dissociate and unfold the inactive complex under the same conditions. The rates of subunit dissociation and unfolding (as monitored by changes in light scattering, tryptophanyl residue fluorescence, and sulfhydryl group reactivity) correspond to the rate of complex dissociation, and these rates indicate a nonfirst order process. These observations suggested that relatively stable intermediates could be present in 6 M guanidine·HCl, altering the pathways of dissociation and unfolding. Evidence that this could be the case was obtained from experiments with a partially inactivated enzyme in which treatments with EDTA and sulfhydryl reagents yielded stable oligomeric species containing 10-11, 8, 6, and 4 subunits. These intermediates are being investigated to obtain information on isologous and heterologous subunit interactions in the dodecamer. In summary, these studies have shown that active-site ligands considerably stabilize intra- and inter-subunit bonding domains.

Preliminary attempts to reactivate mammalian glutamine synthetase (after auto-inactivation with L-methionine-S-sulfoximine, ATP, and Me^{2+}) in crude extracts prepared from bovine brain were unsuccessful, but these efforts will continue after purification of this enzyme.

Studies on the equilibria and kinetics of Mn^{2+}-glutamine synthetase interactions are continuing. Metal ion-dye chelators and pH indicator dyes in conjunction with certain metal ion chelators have been of unique use in these studies. The approaches described in a recent publication from this laboratory are generally applicable to other Mn^{2+}-protein interactions, and Mn^{2+} is an activator of many enzymes from different sources. Manganese ion interactions with high affinity n_1 sites of glutamine synthetase from *E. coli* can be described in terms of a two-state model in which glutamine synthetase exists in two conformations: the relaxed enzyme, GS^R, dominates in the absence of divalent cations and the tightened enzyme, $MnGS^T$, dominates with Mn^{2+} bound to n_1 sites. These two forms can be distinguished by UV difference spectral measurements. A rate constant for the first order release of Mn^{2+} from $MnGS^T$ was recently reported, as was the ratio of rate constants, k_1/k_{-1}, for Mn^{2+} binding to GS^R to form $MnGS^R$. The half-time of the release of Mn^{2+} from $MnGS^T$ is ~ 0.22 second at 15°C; in contrast, the half-time of the overall relaxation process promoted by EDTA ($MnGS^T \rightarrow GS^R$) is > 2 minutes at 15°C. Whereas active-site ligands do not affect the rate of the overall tightening process, $GS^R \rightarrow MnGS^T$, we have found that L-methionine-S,R-sulfoximine and L-glutamate decrease the rates of both reactions involved in the overall relaxation process ($MnGS^T \rightarrow GS^T$ and $GS^T \rightarrow GS^R$). Thus, active-site ligands appear to bind to both $MnGS^T$ and GS^T and to stabilize these forms. Earlier studies of this laboratory showed that L-methionine-S,R-sulfoximine and L-glutamate increase the affinity of n_1 sites of the enzyme for Mn^{2+} ions.

Recently published results on "A novel reaction catalyzed by unadenylylated glutamine synthetase from *E. coli*: AMP-dependent synthesis of pyrophosphate and L-glutamate from orthophosphate and L-glutamine" and "Calorimetric estimate of the enthalpy change for the substrate-promoted conformational transition of aspartate transcarbamoylase from *E. coli*" were summarized in the annual report of this laboratory last year.

Annual Report of the
Section on Metabolic Regulation
Laboratory of Biochemistry
National Heart, Lung, and Blood Institute
October 1, 1980 to September 30, 1981

The research activities of the investigators in the Section on Metabolic Regulation are mainly concerned with the physical and chemical approaches for resolving the mechanisms of enzyme action and its regulation. Currently the research is concentrated on (1) the regulation of enzymatic activity by cyclic cascade system and by Ca(II)·calmodulin complex; (2) elucidating the catalytic mechanism of glutamine synthetase; (3) preparation of monoclonal antibodies against the adenylylated glutamine synthetase and (4) developing analytical methods for biochemical studies. Together, these research programs would provide better understanding on how biochemical processes function in the cells.

I. Regulation of Enzymatic Activity

A. Glutamine Synthetase Cascade: Isolation and Characterization of UT-UR Enzyme

In the bicyclic cascade of glutamine synthetase, uridylyltransferase (UT) and uridylyl-removing enzyme (UR) serve as the converter enzyme for the uridylylation and deuridylylation of a regulatory protein, P_{II}, respectively. The covalently modified and unmodified P_{II} in turn activates the deadenylylation and adenylylation activity of the adenylyltransferase, respectively. Purification of both UT and UR was accomplished using a plasmid-containing strain which contains 25 times higher UT and UR activities than the wild type strain. The purified protein contains both UR and UR activities and it aggregates readily to form dimer, tetramer and a higher molecular weight aggregate. This aggregation appears to be reversible. The monomer was found to be the active species. It has a molecular weight of 92,000-93,000. The amino acid composition of the enzyme was also determined.

B. Phosphorylation-Dephosphorylation Cascade

Theoretical analysis of a monocyclic cascade model reveals that such interconvertible enzyme cascade possesses unusual regulatory properties. To test the validity of the theoretical predictions, a monocyclic cascade was developed. It consists of two converter enzymes, a cyclic-AMP dependent protein kinase and a phosphoprotein phosphatase, and a synthetic substrate peptide, leu-arg-arg-ala-ser-val-ala-gln-leu. Both converter enzymes have been purified to near homogeneity from bovine heart. The affects of some effectors, such as divalent metal ions and ATP, on the activity of the converter enzymes has been characterized. Currently, studies are being carried out with the complete cascade system. It is believed that this cascade system would serve as an excellent model for investigating the regulatory properties of monocyclic cascades.

C. HMG CoA Reductase Cascade: A Theoretical Analysis

In continuation on the theoretical analysis of cyclic cascade system, we extended our analysis to a branched cascade system, 3-hydroxy-3-methylglutamyl (HMG)CoA reductase cascade, which was established mainly by Beg and Brewer and by Gibson and Ingebretsen. Steady-state expression, derived with the assumption that the concentration of the convertor enzyme·interconvertible enzyme complex is negligible with respect to the total concentration of the converter enzyme,

for the fractional unmodified form (active form) of the reductase shows th the specific activity of the reductase is modulated by a multiplicative function involving 24 parameters and 23 of these parameters can be altered by allosteric effectors. The results reveal that (1) the HMG CoA reductase cascade is highly flexible with respect to allosteric regulation; (2) like the straight chain cyclic cascades, the branched cascades also posses strong signal amplification potential; (3) the reductase activity is highly sensitive to the changes in cAMP concentration and this sensitivity is reduced when additional inhibitor for the phosphoprotein phosphatase is incorporated into the cascade.

D. Regulation by Calmodulin

1. Regulation of Calmodulin Dependent Cyclic Nucleotide Phosphodiesterase by Calmodulin and Ca(II) - Calmodulin is known to mediate the effect of Ca(II) regulation. A mechanism, which takes into consideration the interactions between cyclic nucleotide phosphodiesterase and various Ca(II)-liganded calmodulin species, has been proposed for the activation of the calmodulin-regulated cyclic nucleotide phosphodiesterase. Using the steady-state kinetics, we demonstrated that the predominant calmodulin-activated enzyme species contains four Ca(II). Deactivation of the calmodulin-activated phosphodiesterase using either calmodulin binding protein, EGTA or a Ca(II) specific indicator (BAPTA), we have shown that (i) deactivation of phosphodiesterase is mainly accomplished by the removal of Ca(II) instead of Ca(II)·calmodulin complex from the Ca(II)·calmodulin·phosphodiesterase complex, (ii) The $Ca(II)_4$·calmodulin·phosphodiesterase is the active species, (iii) The existence of $Ca(II)_n$·calmodulin·phosphodiesterase complexes where n is 1,2,3 and 4; and the binding of each Ca(II) to the calmodulin-enzyme complex causes it to assume a unique conformational state, (iv) Ca(II) dissociation from $Ca(II)_4$·calmodulin and $Ca(II)_4$·calmodulin·phosphodiesterase proceeds via multiphasic exponential function and the phosphodiesterase appears to slow down each Ca(II) off-rate by a factor of $\sim$100. With the assumption that the stability constant for Ca(II)-calmodulin binding is inversely proportional to the off-rate, one would expect the dissociation constant for the calmodulin·phosphodiesterase complex to be $\sim 10^{-2}$ M; thus, no such complex has been detected.

2. Direct Interaction Between Calmodulin and ACTH - It has been reported that ACTH inhibits phosphodiesterase activation by calmodulin. Kinetics of the deactivation indicates that ACTH forms a complex with calmodulin and thus prevents the formation of calmodulin·phosphodiesterase complex. The formation of ACTH·calmodulin complex was also demonstrated by direct binding study using the fluorescent signal of tryptophan in ACTH.

II. Mechanism of Enzyme Action:

A. Glutamine Synthetase from *E. coli*

1. Catalytic Cycle for the Biosynthesis of Glutamine Catalyzed by the Adenylylated Glutamine Synthetase - The covelently-attached AMP moiety of the adenylylated glutamine synthetase has been replaced by its fluorescent analog, 2-aza-1,N^6-ethano-AMP. The modified glutamine synthetase (aza-ε-G exhibits divalent cation requirement (Mn(II), rather than Mg(II)), pH profile V_{max} and K_m similar to those of naturally adenylylated enzyme. Whereas naturally adenylylated glutamine synthetase exhibits only negligible fluorescence changes upon the binding of substrates, aza-ε-GS exhibits large

fluorescence changes. The fluorescence changes have been used by means of a stopped-flow technique to reveal the involvement of five fluorometrically distinct intermediates in the catalytic cycle for the biosynthesis of glutamine catalyzed by the adenylylated glutamine synthetase. The mechanism is very similar to that previously established for the unadenylylated enzyme, using intrinsic tryptophan fluorescence. Substrates bind via a rapid equilibrium-random mechanism, but the reaction proceeds in a stepwise manner. The formation of an enzyme-bound intermediate (probably γ-glutamyl phosphate + ADP) from ATP and L-glutamate is the rate limiting step, with the subsequent reaction of the enzyme-bound intermediate occurring very rapidly. The success in elucidating this complex mechanism is due largely to the vastly different amplitudes of the fluorescence changes at the two excitation maxima (300 nm and 360 nm) of the aza-ε-AMP moiety which accompany the formation of the various intermediates.

2. Identification of Substrate Induced Protein Conformational Changes in the Adenylylated Glutamine Synthetase - Spin labeled ATP, 2,2,6,6-tetramethylpiperidine-1-oxyl (Tampo) ATP, was used to adenylylate glutamine synthetase. The Tempo-AMP adenylylated enzyme exhibits similar catalytic properties, pH profile, and inhibitor susceptibility to those of the naturally adenylylated enzyme. The ESR signal of the modified enzyme is quenched by the enzyme bound Mn(II) and the extent of the quenching is dependent upon the distances between the nitroxide radical and the two Mn(II) occupied divalent metal ion binding sites. Thus, the ESR data were used to quantitate the changes in distance between the Tempo-AMP and the two divalent metal ion sites as a function of substrate binding. In the absence of substrate, distances between the nitroxide radical at the adenylylation site and Mn(II) at the n_1 (structural site) and n_2 (nucleotide binding site) were determined to be 19 ± 1.5 Å and 16 ± 1.5 Å respectively. Binding of glutamate causes a reduction of 2Å for the distance between n_1 site and the nitroxide group while the distance between n_2 site and the nitroxide moiety is lengthened by 2Å. In the presence of both ATP and glutamate, a condition for which a reaction intermediate is formed, the distances between the adenylyl group and n_1 site and that of the n_2 site are lengthened by 4Å and 3Å, respectively. The stepwise conformational changes were also observed when one monitored the fluorescence of aza-ε-GS as a function of substrate binding.

3. The Kinetic Mechanism of the γ-Glutamyltransferase Activity of Glutamine Synthetase - Steady-state kinetic method was used to study the γ-glutamytransferase activity of the unadenylylated glutamine synthetase in the presence of Mg(II). With NH_3 as product inhibitor, hydrazine as alternative substrate inhibitor and 2-amino-4-phosphorobutyric acid as a deadend competitive inhibitor for glutamine, the kinetic mechanism of the γ-glutamyltransferase reaction was shown to be rapid-equilibrium-random.

B. Mechanistic Study of Rabbit Skeletal Muscle Actomyosin ATPase

The Lynn-Taylor model for the actomysin ATPase suggests that during each ATP hydrosysis cycle, the complex of myosin subfragment-1 with actin must dissociate into S-1·ATP and actin before ATP hydrolysis can occur. However, results of studies carried out in collaboration with E. Eisenberg indicate that ATP hydrolysis step can occur with myosin attached to actin. To accommodate

this finding, a kinetic model was proposed (Biochem 18, 3895 (1979)). Using both fluorescence measurements and the Pi burst, it is possible to demonstrate that the rate limiting step in the catalytic cycle occurs at the same rate whether myosin subfragment-1 is attached to or detached from actin. This rate limiting step takes place after the ATP hydrolysis step, but preceeds the rapid release of inorganic phosphate.

C. The Mechanism of Activation of Proteinase B from Yeast

Holzer and coworkers have proposed a cascade mechanism for the activation of chitin synthase in yeast. In this cascade, chitin synthase is activated by limited proteolysis by proteinase B. Proteinase B is activated by proteinase A which degrades proteinase B inhibitor. Proteinase A is, in turn, activated by proteinase B by degrading proteinase A inhibitor. Proteinase A can also be activated by incubation at pH 5. Consequently, the activation of proteinase B at pH 5 was interpreted as the degradation of proteinase B inhibitor by proteinase A. However, this porposed cascade cannot be sustained by other investigators. In this study, the activation (slow) of proteinase B was shown to be caused by a slow conformational rearrangement. In addition, previous interpretation of SDS activation of proteinase B as SDS induced dissociation of inhibitor from proteinase B, was found to be due to increased solubility of the of the substrate used by SDS instead of dissociation of proteinase B inhibitor.

III. Immunochemical Studies

Preparation of Monoclonal Antibodies Specific to AMP Moiety of Adenylylated Glutamine Synthetase

Antibodies produced against an AMP-bovine serum albumin conjugate exhibit highly specific binding to the AMP moiety of adenylylated glutamine synthetase (Biochem. Biophys. Res. Commun. 82, 865 (1978)). Since the dodecameric enzyme can exist in various states of adenylylation, it provides a unique condition for studying the multideterminant antigen-antibody interaction. In order to obtain homogeneous antibodies, preparation of monoclonal antibodies is currently in progress. Once homogeneous antibodies are available, their interaction with glutamine synthetase at various states of adenylylation is opened to investigation by both physical and immunochemical approaches.

IV. Development of Analytical Methods

A. An Extension of the Theory of Continuous Variation for the Determination of K_d and Stoichiometry

The continuous variation method for determining the binding constant and stoichiometry (also known as Job's plot) has been extended to stepwise binding for identical and noninteracting sites using the limiting-slope of Asmus (Huang, unpublished treatment). The treatment indicates that the stoichiometry can be accurately determined only if the total molar concentration of protein and ligand is sufficiently high relative to the dissociation constant. This limitation was overcome by the present extension of the theory which provides a guide for using the relative magnitudes of protein-ligand complexes at various points to determine the reliability of the data and to estimate the value of K_d. In addition, guidelines are given for using the continuous variation method to determine both K_d and stoichiometry.

B. Synthesis of A Fluorometric Peptide Substrate for cAMP Dependent Protein Kinase and Phosphoprotein Phosphatase

A novel peptide substrate for adenosine 3',5'-monophosphate dependent protein kinase, Leu-Arg-Arg-Trp-Ser-Leu-Gly, has been synthesized. Phosphorylation of the peptide causes a 20% increase in the peptide fluorescence intensity at 358 nm. Values of K_m and k_{cat} for the phosphorylation reaction at pH 7.0, 25°C, were determined to be 2.7 ± 0.5 uM and 5.5 ± 0.4 sec^{-1}, respectively. The phosphorylated peptide was shown to be an effective substrate for phosphoprotein phosphatase with a K_m of 113 ± 10 uM and a k_{cat} of 2.4 ± 0.2 sec^{-1} in the presence of 2.5 mM $MnCl_2$. Changes in the peptide fluorescence intensity was shown to be a function of its phosphorylation state. The fluorescence peptide can detect phosphorylation changes in the picomole range and the changes in fluorescent signal can be observed and continuously recorded within milliseconds.

C. Determination of Divalent Metal Ion Binding Constant to A Ca(II) Specific Indicator

A Ca(II) specific indicator, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N',-tetraacetic acid (BAPTA) was synthesized as described (Biochem 19, 2396 (1980)). Binding of Ca(II), Mg(II), Ni(II), Zn(II) or Mn(II) to BAPTA causes a significant spectral change. This signal was used to determine the dissociation constant for CaBAPTA, NiBAPTA and ZnBAPTA, which is 6.25 x 10^{-7}, 5.7 x 10^{-8} and 1 x 10^{-9} M, respectively. Although BAPTA was originally thought to be a Ca(II) specific indicator, it is in fact a good indicator for many divalent metal ions.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00201-10 LB

PERIOD COVERED
October 1, 1980 to September 30, 1981

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

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

PHS-6040
(Rev. 2-81)

Project Description

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

Major Findings

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

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

The first step is catalyzed by the enzyme, leucine 2,3-aminomutase, which requires coenzyme B_{12} [adenosylcob(III)alamin]. This enzyme has been partially purified from a variety of sources: clostridia, spinach, potatoes, and sheep liver. It has been measured in human liver, hair roots, and leukocytes.

The intracellular distribution of the enzyme, leucine 2,3-aminomutase, has been examined in rat liver. It is primarily cytosolic, although there is a minor amount of activity in washed mitochondria. The activity is found widely distributed in the organs of the rat. Muscle tissue (heart and skeletal) is rich in activity as is the brain. Kidney has somewhat less activity per mg protein than liver.

In a continuing study, sera from patients with pernicious anemia have been examined. It has become clear that the lowered amounts of cobalamin in these deficient individuals cause a decrease in the circulating levels of leucine accompanied by an increase in the levels of β-leucine. When these deficient individuals are given an injection of cobalamin, the levels of leucine begin to rise while those of β-leucine fall to normal. Interestingly, the levels of circulating valine, which are normal in the pernicious anemia patients, undergo a transient decline during the same time the changes are noted in the leucine and β-leucine levels. This is consistent with the metabolic pathway observed in rat livers:

$$\text{valine} \rightleftharpoons \alpha\text{-ketoisovalerate} \rightarrow \text{isobutyryl-CoA} \rightarrow \rightarrow \rightarrow \text{methylmalonyl-CoA} \underset{}{\overset{B_{12}}{\rightleftharpoons}} \text{succinyl-CoA}$$

$$\text{leucine} \overset{B_{12}}{\rightleftharpoons} \beta\text{-leucine} \rightleftharpoons \beta\text{-ketoisocaproate}$$

(β-ketoisocaproate → isobutyryl-CoA + acetate)

When the two cobalamin-dependent enzymes, methylmalonyl-CoA mutase and leucine 2,3-aminomutase, are activated by the addition of their cofactor, the pool of valine is subjected to a double draining. It seems to take these

individuals about a week to recover their normal circulating levels of valine.

The enzyme which converts β-leucine to β-ketoisocaproate, β-leucine transaminase, has been examined in sheep liver. An assay has been developed in which the product of the reaction, β-ketoisocaproate, is reduced to β-hydroxyisocaproate at the expense of NADH. This uses the commercially available coupling enzyme, β-hydroxybutyrate dehydrogenase from Pseudomonas lemoigne. The coupling enzyme has a K_m for β-ketoisocaproate of 1×10^{-4} which compares well with the reported value of 7×10^{-4} M for its natural substrate, β-ketobutyrate.

Attempts to purify the transaminase have shown that the activity is contained in a high molecular weight material. When this is treated with Titron X100, the activity has a molecular weight of 208,000. Attempts to demonstrate the purity of this latter material by gel electrophoresis have been frustrated by the tendency of the activity to aggregate in oligomeric structures with molecular weights ranging from 400,000 to 1,000,000.

The transaminase is competitively inhibited by cycloserine with an apparent K_i of 2 mM. It has an apparent K_m for DL-β-leucine of 4 mM and that for α-ketoglutarate is 1.7 mM. The transaminase has an absolute requirement for α-ketoglutarate; neither pyruvate nor oxalacetate will support the reaction. L-leucine will replace DL-β-leucine with an apparent K_m of 2 mM.

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 tissue will be studied as will its relation to plant development. The relation of the pathway to the metabolism of other amino acids and to lipids will be examined. The other enzymes in the pathway will be examined and their cofactor requirements will be 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 material and turn over cellular components; and (3) the probable nutritional value of plant material with regard to vitamin B_{12}. 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, isovalericacidemia, 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.: Distribution of leucine 2,3-aminomutase activity in various organs of the rat and in subcellular organelles of rat liver. Biochem. Biophys. Res. Commun. 96: 838-843, 1980.

Poston, J.M.: Cobalamin-dependent formation of leucine and β-leucine by rat and human tissue. Changes in pernicious anemia. J. Biol. Chem. 255: 10067-10072, 1980.

Poston, J.M.: The cobalamin-dependent interrelation of valine, leucine, and β-leucine in the rate and human. In Walser, M. and Williamson, J.R. (Eds.): Developments in Biochemistry, Vol. 19, International Symposium on Metabolism and Clinical Implications of Branched-Chain Amino and Ketoacids, Kiawah Island Conference Center, Charleston, South Carolina, 1980. New York, Elsevier/North-Holland, 1981, 401-404 pp.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00202-10 LB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Kinetics, Regulation and Mechanism of Biochemical Reactions

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

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Study of calmodulin (CM)-regulated cyclic nucleotide phosphodiesterase (PDE) reveals that (i) deactivation of the CM-activated PDE is achieved by the removal of one Ca(II) from the Ca(II)·CM·PDE complex. This is consistent with the proposal that $Ca(II)_4$·CM·PDE is the active species; (ii) the existence of $Ca(II)_n$·CM·PDE where n is 1, 2, 3, and 4; (iii) Ca(II) dissociation from $Ca(II)_4$·CM and $Ca(II)_4$·CM·PDE proceeds via multiphasic exponential function, and complexation with PDE causes each Ca(II) off-rate by a factor of ~ 100. (2) A fluorescent peptide has been synthesized. This peptide is shown to be a good substrate for cAMP-dependent protein kinase and its phosphorylated form serves as a substrate for phosphoprotein phosphatase. (3) Theoretical analysis of cyclic cascade model was extended to a branched cascade system, the HMG CoA reductase cascade. (4) Study of a spin-labeled Tempo-adenylylated glutamine synthetase revealed changes in distances between the adenylyl site and the two metal ion sites due to substrate binding and formation of an intermediate. (5) Kinetics and thermodynamics of divalent metal ion binding to a Ca(II) specific indicator were studied. The mechanism of actomyosin ATPase was investigated.

PHS-6040
(Rev. 2-81)

Project Description

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

Major Findings

I. Mechanism of Activation of Calmodulin-Regulated Cyclic Nucleotide Phosphodiesterase (also see Annual Report of C.Y. Huang).

Calmodulin plays an important role in mediating the effect of Ca(II) in cellular regulation. A mechanism for calmodulin-regulated activation of cyclic nucleotide phosphodiesterase has been reported (Proc. Natl. Acad. Sci. 78, 871, 1981). In this proposed mechanism, we take into consideration the interactions between the cyclic nucleotide phosphodieserase and various Ca(II)-liganded calmodulin species. The result of the steady-state kinetic study showed that the predominant calmodulin-activated enzyme species contains four Ca(II). To further characterize the activation mechanism, we carried out kinetic studies on the deactivation reaction. As previously reported, deactivation by dissociating the calmodulin·$Ca(II)_4$ complex from the Ca(II)-calmodulin-phosphodiesterase complex is relatively slow, with a rate constant of 3.2×10^{-3} sec^{-1} at 25°C. The kinetics of deactivation by Ca(II) removal using EGTA was studied using a three-syringe stopped-flow machine. The data show that the rate of deactivation proceeds via a single exponential function with a rate constant of 4.5 sec^{-1} at 25°C, a rate which is 1,400-fold faster than that by dissociating $Ca(II)_4$-calmodulin complex from the enzyme-activator complex using calmodulin binding protein. This deactivation rate was compared with the Ca(II) off-rate from the Ca(II)·calmodulin·phosphodiesterase complex. To obtain this rate, we first investigated the Ca(II) off-rate from Ca(II)·calmodulin complex. The release of Ca(II) was monitored (1) by the tyrosine fluorescence changes accompanying the Ca(II) binding and (2) by means of a Ca(II) specific indicator, 1,2-bis(*o*-aminophenoxyl)ethane-*N*,*N*,*N*',*N*'-tetraacetic acid, which was synthesized by Dr. L. Tsai. In both cases, one observed only a single exponential function with an identical rate constant, 12 sec^{-1} at 25°C. Since calmodulin binds four Ca(II) with different affinity, we expect that the release of Ca(II) must proceed with a multiple exponential function. It is reasonable to assume that the Ca(II) off-rate is too fast for the stopped-flow machine to pick up. This reasoning was supported by the fact that when the indicator concentration was reduced significantly, a multiple exponential function for the Ca(II) release was observed.

When the Ca(II) released from the Ca(II)·calmodulin·phosphodiesterase complex was studied using the Ca(II) specific indicator, we observed a multiple exponential function for the Ca(II) off-rate, and the fastest step has a rate constant between 3.5-4.6 sec^{-1}, which is in reasonable agreement with the 4.5 sec^{-1} determined for

the deactivation rate. The slowest step has a rate constant of ~ 0.1 sec^{-1}. The results of this study indicate that (1) since deactivation of phosphodiesterase by Ca(II) removal is ~ 1,400-fold faster than the Ca(II)·calmodulin off-rate, it is likely that deactivation of phosphodiesterase _in vivo_ is accomplished by the removal of Ca(II) from the enzyme-bound calmodulin; (2) the fact that both deactivation via Ca(II) removal and the fastest Ca(II) off-rate monitored by a Ca(II) specific indicator has the same rate constant, and the time course for the deactivation proceeds via a single exponential function indicates that deactivation occurs once a Ca(II) is dissociated from the enzyme-bound calmodulin. This is in accord with the hypothesis that four Ca(II) are required for the calmodulin activation of phosphodiesterase; (3) since the Ca(II) released from the enzyme-bound calmodulin is multiphasic, it demonstrates the existence of EAC_3, EAC_2, and EAC [E = phosphodiesterase, A = calmodulin, and C = Ca(II)], at least in the transient phase. In addition, each Ca(II) off-rate from the enzyme-bound calmodulin is slower by a factor of about 100 relative to its corresponding off-rate from the Ca(II)·calmodulin complex. This indicates that binding of each Ca(II) to the enzyme-calmodulin complex causes it to assume a different conformational state; (4) with the assumption that changes in Ca(II) binding are mainly due to the variation of its off-rate, one would expect the dissociation constant for the calmodulin·phosphodiesterase complex to be ~ 10^{-2} M. Thus, it explains why no calmodulin-phodiesterase complex has been detected.

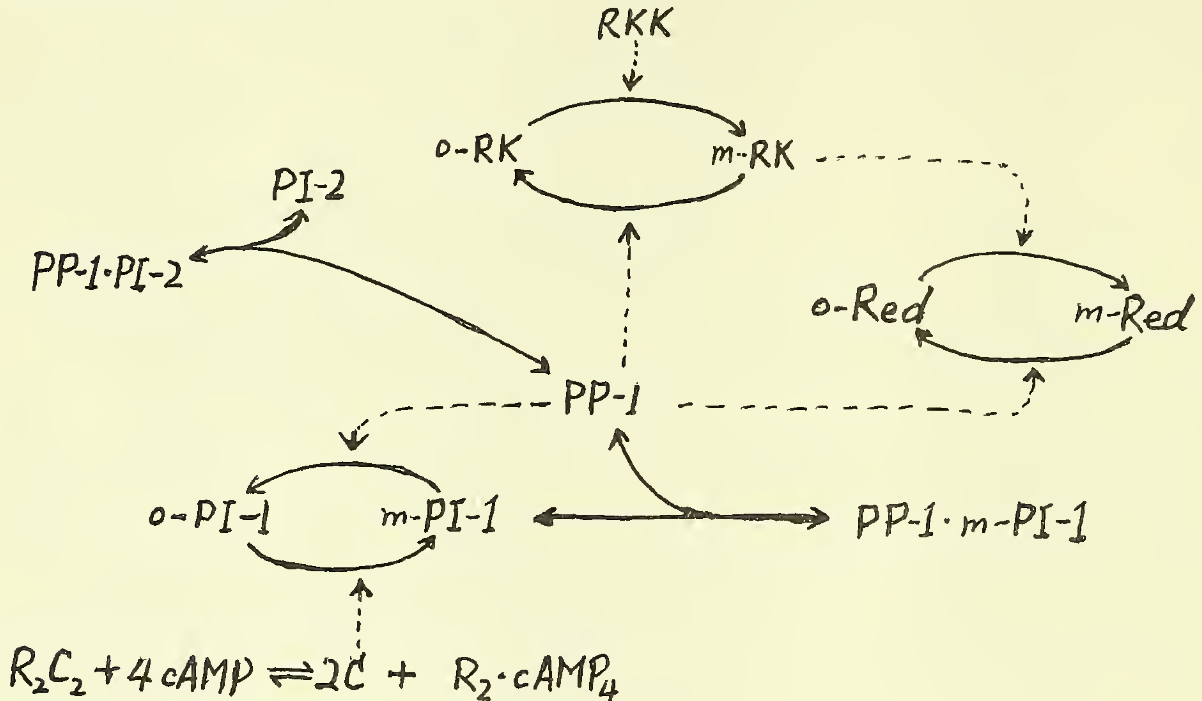
II. Synthesis of a Fluorometric Peptide Substrate for cAMP-Dependent Protein Kinase and Phosphoprotein Phosphatase

The use of [γ-^{32}P]ATP and [^{32}P]-phosphorylated peptide to monitor the activity of cAMP-dependent protein kinase and phosphoprotein phosphatase, respectively, provides severe constraints on the methodology that can be used for detailed physico-chemical study of the enzymatic reaction. An alternative method of assay is therefore highly desirable. Recently, Bramson _et al._ (J. Am. Chem. Soc. _102_, 7156, 1980) found that phosphorylation of a substrate peptide containing a nitrotyrosine residue adjacent to the serine phosphorylation site causes a measurable spectral change at 430 nm and can be used for the continuous assay of protein kinase activity. We have synthesized a peptide, Leu-Arg-Arg-Trp-Ser-Leu-Gly, which can be served as an effective substrate for the protein kinase. Phosphorylation of this peptide causes a 20% increase in the peptide fluorescence intensity at 358 nm with the excitation wavelength set at 280 nm. Values of K_m and k_{cat} for the phosphorylation reaction at pH 7.0, 25°C were determined to be 2.7 ± 0.5 µM and 5.5 ± 0.4 sec^{-1}, respectively. The phosphorylated peptide is an effective substrate for phosphoprotein phosphatase with a K_m of 113 ± 10 µM and k_{cat} of 2.4 ± 0.2 sec^{-1} in the presence of 2.5 mM $MnCl_2$. Changes in the peptide fluorescence intensity were demonstrated to be a function of its phosphorylation state. This fluorescence signal allows one to detect phosphorylation changes in the picomole range, comparable to the sensitivity of the common assays which use radioactive phosphate. In addition, the fluorescence changes resulting from phosphorylation and dephosphorylation of this peptide can be observed and continuously recorded within milliseconds. Thus, the fluorescence peptide provides a new dimension for studying the mechanism of action of cAMP-dependent protein kinase and phosphoprotein phosphatase.

III. Covalent Interconvertible Enzyme Cascade and Metabolic Regulation

In our earlier work, we have demonstrated through theoretical and experimental analysis that cyclic cascade systems, derived from the dynamic coupling of two opposing cascades involving covalent modification and demodification of enzyme(s)

or protein(s), can provide enormous signal amplification, rate amplification, and flexibility for metabolic regulation of enzymic activity. Recently, we carried out theoretical analysis for a branched cascade system, 3-hydroxy-3-methylglutaryl (HMG) CoA reductase cascade, which was established mainly by Beg and Brewer and by Gibson and Ingebretsen. This HMG CoA reductase catalyzed the NADPH-dependent reduction of HMG to mevalonate which is believed to be the rate limiting step for the cholesterol biosynthesis in rat liver. The cascade which regulates this enzyme is shown below:



The unmodified form of the reductase (o-Red) is active while the phosphorylated form (m-Red) is inactive. Phosphorylation of the reductase is catalyzed by the phosphorylated form of reductase kinase (m-RK), while the phosphorylation of the unmodified reductase kinase (o-RK) is catalyzed by the reductase kinase kinase (RKK). Dephosphorylation of both reductase and reductase kinase is catalyzed by phosphoprotein phosphatase (PP-1), which is inactivated by the phosphorylated form of the phosphatase inhibitor 1 (m-PI-1) and also inhibited by phosphase inhibitor 2 (PI-2). Phosphorylation of the phosphatase inhibitor 1 is catalyzed by the catalytic subunit (C) of the cAMP-dependent protein kinase (R_2C_2). Steady-state expression, derived with the assumption that the concentration of the converter enzyme·interconvertible enzyme complex is neglibible with respect to the total concentration of the converter enzyme, for the fractional unmodified form of the reductase shows that the specific activity of the reductase is modulated by a multiplicative function involving 24 parameters, and 23 of these parameters can be altered by allosteric effectors. The results of the computer simulation reveal that: (1) the HMG CoA reductase cascade is highly flexible with respect to allosteric regulation; (2) like the straight chain cyclic cascade system, this branched cascade system also possesses strong signal amplification; (3) in the absence of phosphoprotein phosphatase inhibitor 2, the reductase activity is highly sensitive to the changes in cAMP concentration. This sigmoidal response is

derived from the fact that active phosphoprotein phosphatase dephosphorylates both the reductase and reductase kinase. However, this sensitivity with respect to cAMP concentration can be significantly reduced when the phosphoprotein phosphatase inhibitor-2 is introduced into the system. Thus, by introducing multiple inhibitors for the phosphoprotein phosphatase, it results in a decrease in sensitivity toward changes in effector concentration.

IV. Identification of Substrate Induced Protein Conformational Changes in Adenylylated Glutamine Synthetase

Spin-labeled ATP, 2,2,6,6-tetramethylpiperidine-1-oxyl(Tempo)ATP, was used to adenylylate the E. coli glutamine synthetase. The Tempo-AMP adenylylated enzyme exhibits similar catalytic properties, pH profile, and inhibitor susceptibility to those of the naturally adenylylated enzyme. The ESR spectrum of the spin-labeled enzyme is very similar to the spectrum of free Tempo-ATP, indicating that the Tempo moiety of the adenylylated enzyme is only slightly immobilized and possesses a rotational correlation time of less than 8×10^{-10} second. This observation suggests that the adenylylation site is located on the surface of the enzyme. The ESR signal is quenched by the enzyme bound Mn(II) and the extent of the quenching is dependent upon the distances between the nitrooxide radical and the two Mn(II) binding sites. This property was utilized to quantitate the protein conformational change induced by substrate binding. In the absence of substrate, the distance between the Tempo-adenosine moiety and the structural divalent metal ion binding site (n_1 site), and the catalytic site (n_2 site) were determined to be 19 $\pm$ 1.5 and 16 $\pm$ 1.5 Å, respectively. Titration data showed that substrates exert a significant effect on the binding affinity of Mn(II). This effect is especially pronounced at the n_2 site. For example, at a total Mn(II) concentration of 9.09×10^{-5} M, only 3% of the n_2 sites are filled in the absence of substrates, in contrast, the fractional saturation increases to 21% in the presence of glutamate and ATP, and to 44% in the presence of ADP and glutamine. In addition, from the fractional saturation of Mn(II) on the n_1 and n_2 sites, and the extended ESR signal quenching, we can estimate the change in distances between the spin-labeled moiety and the n_1 and n_2 sites. The results show that the distance between n_1 site and the spin-labeled AMP changes from 19 $\pm$ 1.5 Å in the absence of substrate to 17 $\pm$ 1.5 Å in the presence of glutamate, and to 21 $\pm$ 1.5 Å in saturating ATP and glutamate. The distance between n_2 site and the Tempo-adenylyl group varies from 16 $\pm$ 1.5 Å in the absence of substrate to 18 $\pm$ 1.5 Å in the presence of glutamate to 21 $\pm$ 1.5 Å in the presence of both glutamate and ATP. It is interesting that binding of glutamate induces a protein conformational change such that the distance between n_1 site and Tempo-adenylyl group is shortened by ~ 2 Å. However, in the presence of both ATP and glutamate, a condition for which a reaction intermediate is formed, an additional protein conformational change is observed by the fact that the n_1 site to Tempo-AMP distance is lengthened by ~ 4 Å and the n_2 site to Temp-AMP distance is also lengthened by ~ 3 Å. These stepwise changes in the protein conformation are consistent with the observed fluorescence changes described previously and also consistent with the report that Mn(II) binding affinity at the n_1 site is enhanced by L-glutamate (Shrake et al., Biochemistry 16, 4372, 1977). Binding of products such as ADP and Pi, ADP, and glutamine also exerts significant effect on the ESR signal of the nitrooxide radical. Qualitatively speaking, binding of these products tends to shorten the n_1 site to Tempo-AMP distance and lengthen the distance between n_2 site and Tempo-AMP.

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

The Lynn-Taylor model for the actomyosin ATPase (Biochemistry 10, 4617, 1971) suggests that ATP hydrolysis required the dissociation of ATP·actomyosin complex to ATP·myosin and actin. This proposed mandatory detachment step was based on the observation that, at low actin concentration, ATP causes dissociation of the acto-heavy meromyosin complex prior to ATP cleavage. In contrast to the Lynn-Taylor model, which predicts the rate of initial Pi burst should remain constant or decrease at high actin concentration, we found that this rate increases markedly at high actin concentration. In addition, at high actin concentration, the magnitude of the initial Pi burst is much larger than is predicted by the Lynn-Taylor model. Furthermore, at 360 μM actin, at which more than 90% of the S-1·ATP is bound to actin, there is no inhibition of the steady-state ATPase activity contrary to the 70% inhibition predicted by the Lynn-Taylor model. These observations together with the initial Lynn-Taylor observation indicate that S-1·ATP is in rapid equilibrium with acto-S-1·ATP and that because the tight binding of ATP greatly reduces the affinity of S-1 for actin, the equilibrium is shifted toward S-1·ATP at low actin concentration. At high actin concentration, the equilibrium is shifted toward acto-S-1·ATP, and the initial Pi burst is faster for acto-S-1·ATP than for S-1·ATP. These findings are consistent with a mechanistic scheme reported earlier (Stein *et al.*, Biochemistry 18, 3895, 1979) which does not include a mandatory detachment step. In other words, myosin does not have to detach from actin during each cycle of ATP hydrolysis since the ATP hydrolysis can occur with myosin attached to actin. Using both fluorescence measurements and direct measurements of the Pi burst, we found that the rate-limiting step in the catalytic cycle occurs at the same rate whether S-1 is attached to or detached from actin. This rate-limiting step takes place after the ATP hydrolysis step, but precedes the rapid release of Pi.

VI. Determination of Divalent Metal Ion Binding Constant to 1,2-bis(o-aminophenoxy)ethane-N,N,N', N',-tetraacetic Acid

A Ca(II) specific indicator, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid (BAPTA) was synthesized as described (Biochemistry 19, 2396, 1980). This compound was found to be a good indicator for Ca(II), Mg(II), Mn(II), Ni(II), and Zn(II). In each case, binding of divalent metal ion causes a significant change in the UV spectrum of the compound. Direct determination of the binding constant was complicated by the fact that BAPTA binds very tightly with divalent metal ion. In order to resolve this problem, we first determined the binding constant for Ni(II) using the rapid kinetic technique because Ni(II) has a relatively slow H_2O exchange rate and the NiBAPTA spectrum is significantly different from that of CaBAPTA or ZnBAPTA. The rate constant for the binding of Ni(II) to BAPTA was determined to be $2.46 \times 10^5\ M^{-1}\ sec^{-1}$ at 25°C and its dissociation rate constant is $1.4 \times 10^{-2}\ sec^{-1}$, thus, the dissociation constant for NiBAPTA is 5.7×10^{-8} M. NiBAPTA complex was used to determine the dissociation constant for CaBAPTA, which was evaluated to be 6.25×10^{-7} M at 25°C. The rate constant for Ca(II) dissociation is 190 sec^{-1}. From the K_d and the dissociation rate constant, one can calculate the rate constant for Ca(II) binding which is ~ $3 \times 10^8\ M^{-1}\ sec^{-1}$. This value is in reasonable agreement with the rate constant for substitution of inner sphere H_2O for Ca(II). Using a similar method, the dissociation constant for BAPTA was determined to be 1×10^{-9} M.

Significance to Biomedical Research

The overall objective is to gain a better understanding of how enzymes function with respect to their catalytic and regulatory properties, and to elucidate principles of interaction between effectors, regulators, and proteins. This knowledge is instrumental in controlling the function of a specific enzyme by designing an effector or enzyme suicide substrate. The study on calmodulin activation of cyclic nucleotide phosphodiesterase can reveal a general mechanism for calmodulin-dependent Ca(II) regulation of enzymic activities.

Proposed Course of Research

(1) To further expand the cyclic cascade model to substrate cascade and more complex enzyme cascade systems; and to further analyze the kinetic aspect of the cyclic cascade system.

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

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

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TITLE OF PROJECT (80 characters or less)

Cellular Regulation of Enzyme Levels

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

The purpose of this investigation is to study the regulation of intracellular protein turnover in E. coli and to isolate and characterize an in vitro cell-free system which catalyzes the degradation of glutamine synthetase (GS). The current studies indicate that degradation of GS by E. coli extracts might be a two-step process consisting of inactivation followed by proteolysis. The inactivation reaction was first observed in extracts of nitrogen starved Klebsiella aerogenes (Fulks, R.M. (1977) Fed. Proc. 36, 3420). Similar inactivation occurs when GS is incubated with ascorbate (Levine, R.L. (1980) Fed. Proc. 39, 401) and with catalase-free E. coli extracts. This reaction requires Fe^{3+}, NADPH, O_2 and is inhibited by catalase. Rabbit liver microsomal P_{450} mixed function oxidase system consisting of cytochrome P_{450} and NADPH cytochrome C reductase catalyzes a similar inactivation and this system has been used as a model to study inactivation without proteolysis. Techniques used in these studies have included polyacrylamide pore gradient electrophoresis, isotopic labeling, chromatographic techniques, autoradiography, enzymatic assay of functional proteins, high performance liquid chromatography.

PHS-6040
(Rev. 2-81)

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 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 degradation or its regulation. The object of this investigation is to elucidate some of the basic mechanisms underlying the process of protein degradation. The current studies have suggested that GS degradation is a two-step process involving inactivation followed by proteolysis. This report is concerned primarily with the initial step, inactivation.

Major Findings

It has been exceedingly difficult to isolate a bacterial cell-free system capable of catalyzing the degradation of specific enzymes in response to nutritional conditions. Early studies undertaken by Fulks in this laboratory had demonstrated a rapid inactivation of GS followed by loss of immunological cross reactivity in situ in K. aerogenes under conditions of nitrogen limitation (Fulks, R.M. (1977) Fed. Proc. 36, 3420). A similar GS inactivation occurred in extracts of the same cells but without subsequent loss of immunological cross reactivity. These results suggested a two-step process for GS degradation consisting of initial inactivation followed by proteolysis.

Further studies revealed that GS inactivation by K. aerogenes extracts required iron (III) molecular oxygen, NAD(P)H and was inhibited by catalase, manganese (II) and chelating agents such as EDTA and o-phenanthroline. Then a nonenzymatic model ascorbate system was found to catalyze a similar GS inactivation (Levine, R.L. (1980) Fed. Proc. 39, 401). The inability to detect GS inactivation in E. coli extracts prompted an examination of the endogenous catalase levels in the two organisms, E. coli and K. aerogenes

It was found that although E. coli extracts possessed high catalase levels, K. aerogenes was nearly devoid of catalase activity. It should be noted that K. aerogenes normally possesses catalase activity but the strain of K. aerogenes used in these studies lacked detectable catalase activity.

These results suggested that E. coli preparations lacking catalase activity might possess the capacity to catalyze GS inactivation and therefore GS degradation. A catalase-deficient strain of E. coli was obtained by a neomycin selection technique which was based on the observation that a high proportion of neomycin resistant mutants are heme-deficient and therefore catalase-deficient. Preliminary studies indicated that extracts of catalase-deficient mutants were unable to catalyze GS degradation as determined by SDS pore gradient electrophoresis. Although at the time an explanation for this observation was not readily apparent, recent studies have revealed at least two contributing factors. First, catalase levels in wild type E. coli are normally approximately 27.5 units/mg extract protein. The catalase-deficient mutants possess low but detectable catalase levels 0.3-1.8 units/mg extract protein. Under these conditions, 0.1-0.2 units/mg extract protein is sufficient to inhibit GS inactivating activity. When the extracts were partially purified to remove residual catalase, the resulting "catalase-free" preparations catalyzed both GS inactivation and proteolysis. In the second place, the relative yield of GS inactivating activity in catalase-free extracts from catalase-deficient strain is twenty

to sixty-fold lower than comparably prepared extracts from wild type cells. GS inactivating activity has also been observed in catalase-free extracts of Pseudomonas putida. The GS inactivation observed with catalase-free extracts from catalase-deficient strain is qualitatively similar to the GS inactivation characterized in K. aerogenes extracts and the ascorbate model system. Although some minor differences are noted.

We have recently demonstrated GS inactivation in situ in E. coli grown aerobically and anaerobically, as well as in the catalase-deficient strain grown anaerobically. The kinetics of the reaction suggest a first order process and the $t_{0.5}$ of GS in the three cultures is 5.33, 1.21, and 0.65 hours, respectively. Catalase levels in extracts from these same cultures are 27.5, 1.8, and 0.3 units/mg extract protein.

For these in situ studies, frozen cell pastes were first washed in glucose minimal medium and then resuspended in the same medium without ammonia nitrogen. The GS inactivation required iron (III) molecular oxygen and was inhibited by manganese(II). Inactivation was partially inhibited by dinitrophenol (DNP), an inhibitor of electron transport chain linked oxidative phosphorylation, and completely inhibited by arsenate, an inhibitor of substrate phosphorylation. Omission of glucose prevented GS inactivation. These results suggest that metabolic energy is required for GS inactivation, and presumably in the form of ATP. The requirement for iron (III) oxygen and inhibition by manganese suggested that the GS inactivation observed in situ is similar and possibly identical to the inactivation originally characterized in extracts of K. aerogenes.

It was indeed surprising to observe GS inactivation in situ in cultures which yielded extracts possessing high catalase levels, a potent inhibitor in vitro. There are several possible explanations for this observation: (a) catalase activity might be compartmentalized with respect to GS inactivating activity; for example, catalase might be in the soluble fraction and GS inactivating activity might be membrane associated; (b) catalase level or activity might be tightly regulated in vivo; and (c) catalase might not be a natural inhibitor of the inactivation process in vivo. Further studies are necessary to investigate these possibilities.

Because GS inactivation resembles some known mixed function oxidation type reactions, a highly purified reconstituted microsomal cytochrome P_{450} mixed function oxidation system was obtained from M. J. Coon (University of Michigan) and tested for the capacity to catalyze GS inactivation. As noted in the previous report, the microsomal cytochrome P_{450} mixed function oxidation system catalyzes GS inactivation and this reaction is qualitatively similar to the GS inactivation characterized in bacterial cell extracts and the ascorbate model system. It was important to determine whether this GS inactivation reaction was a general property of mixed function oxidation systems and whether a physiologically different and unrelated mixed function oxidation system could catalyze the same reaction. A highly purified reconstituted mixed function oxidation system from camphor grown P. putida was obtained from I.C. Gunsalus (University of Illinois) and tested for the capacity to catalyze GS inactivation.

This mixed function oxidation system is composed of three components, putidaredoxin reductase (PR), an iron sulfur electron carrier protein putidaredoxin (Pd), and cytochrome $P_{450\ cam}$. Our studies showed that PR·Pd catalyzed GS inactivation in the absence of $P_{450\ cam}$. The reaction required molecular oxygen, iron (III) NADH, and was inhibited by catalase manganese (II) and

chelating agents. In this reaction, however, relatively high concentrations of the electron carrier protein Pd were required.

A qualitatively similar GS inactivation was catalzyed by PR·Pd·$P_{450\ cam}$. GS inactivation in this case was stimulated by $P_{450\ cam}$ at lower Pd concentrations. Some minor differences were noted in the inactivation reactions catalyzed by the two mixed function oxidation systems from P. putida. Low concentrations of superoxide dismutase (SOD) had no effect on either inactivation reaction. However, very high concentrations of SOD significantly inhibited the PR·Pd reaction compared to the PR·Pd·$P_{450\ cam}$-mediated GS inactivation. Both DMSO and histidine inhibited GS inactivation in the absence of $P_{450\ cam}$ and exhibited only slight inhibition in the $P_{450\ cam}$-dependent reaction. Mannitol inhibited both PR·Pd and PR·Pd·$P_{450\ cam}$ reactions about 15%. These data suggest the possibility that slightly different pathways of oxygen activation, or different activated oxygen species, are responsible for GS inactivation in the two systems. Such data, alternatively, might reflect the relative affinity of the activated oxygen species for site or sites on the enzyme surface, or a combination of these factors.

In the case of the microsomal P_{450} system, the PR·Pd·$P_{450\ cam}$ system, the ascorbate system, or the K. aerogenes extracts, excess iron (III) did not relieve inhibition of GS inactivation by EDTA, whereas the reverse was observed for the E. coli extracts and the PR·Pd system. These results, again, suggested the possibility of more than one pathway for GS inactivation, a P_{450} or "cytochrome" mediated pathway and/or a PR·Pd or "feredoxin" mediated pathway. It must be remembered that E. coli extracts used for these studies were derived from a heme-deficient, catalase-deficient strain. Although this strain is leaky with respect to heme synthesis, it is possible that the predominant inactivation pathway in crude extracts is a PR·Pd or "feredoxin" like activity. Further studies comparing the inactivating activity in extracts from wild type cells and catalase-deficient cells are needed to clarify these observations.

Studies by Levine have suggested that GS inactivation is the result of a site specific modification of a histidine (Levine, R.L. (1981) Fed. Proc. 40, 871). In various systems, the requirement for iron (III), a reducing system and molecular oxygen seemed to indicate that the reduction and reoxidation of iron was involved. Therefore, studies were undertaken to test the capacity of each of the enzymatic systems to reduce iron (III). The complete microsomal cytochrome P_{450} system and the two P. putida systems, PR·Pd and PR·Pd·P_{450} reduce iron (III) in a NAD(P)H-dependent reaction. These results suggested that GS inactivation is a multi-step process involving (a) reduction of iron (III), (b) binding of iron (II) to divalent cation site of GS, (c) in situ autooxidation of iron (II) to (III) with the generation of one or more activated oxygen species, and (d) in situ oxidation of a susceptible histidine which is associated with the GS active site. Based on the process as outlined, iron (II) alone without a reducing system would be expected to inactivate GS. In fact, Levine has recently demonstrated that iron (II) in a potent nonenzymatic inactivating agent. Also, one would predict, based on the scheme above, that NADH·diaphorase, a flavoprotein capable of reducing iron (III), would constitute the simplest enzymatic reducing system capable of GS inactivation. In fact, diaphorase inactivates GS in a NAD(P)H-dependent reaction.

The physiological aspects of these studies are also important and should be emphasized. In the case of the in situ experiments with K. aerogenes and E. coli, GS inactivation is provoked under specific nutritional conditions, namely, nitrogen starvation. Moreover, in three of the in vitro inactivating systems,

the inactivation reaction is influenced by the state of adenylylation of the GS and by the availability of substrates. In the ascorbate system, the microsomal cytochrome P_{450} system and the PR·Pd system, the unadenylylated GS (physiologically active form) is rapidly inactivated in the absence of its cosubstrates ATP and glutamate, but these same substrates protect the unadenylylated GS from inactivation and accelerate the inactivation of the adenylylated GS (physiologically inactive form). Thus, when glutamine is required in the cell and there is an ample supply of substrates, the active GS is protected from inactivation. If there is a sudden depletion of ATP and glutamate, the unadenylylated enzyme is rapidly inactivated. Neither substrate alone has much of an effect. These data support the contention that this GS inactivation reaction is important for the regulation of enzyme level.

Proposed Course of Action

Many of the observations summarized here are preliminary in nature and further studies are needed for clarification. There are perhaps four areas which warrant intensive investigation.

First, isolation, purification, characterization and *in vitro* reconstitution of the GS inactivating and proteolytic activity is planned. Although the proteolytic activity will receive priority, previous observations suggested that GS inactivating and proteolytic activities copurify, and it is possible that the two activities are coupled. These studies are complicated by the possibility that multiple components are involved and that inactivating activity cannot be determined until residual catalase activity is removed. Because ascorbate inactivated GS is more susceptible to proteolysis by *E. coli* crude extracts, the preparation of labeled ascorbate-inactivated GS would be substrate of choice for screening the proteolytic activity during purification. In addition, subcellular localization studies might also facilitate purification if there is compartmentation of the GS inactivating or proteolytic activities with respect to each other, or the inactivating activity with respect to catalase. Also, it would be appropriate to compare the GS inactivating activity from *P. putida*, *E. coli* and heme-deficient strains in order to clarify some of the observed differences.

The second area of investigation is the systematic study of microsomal cytochrome P_{450} inactivation of GS. The rationale for such a study is two-fold. First, there are perhaps eight to twenty isozymes of cytochrome P_{450} in liver microsomes. These enzymes exhibit overlap of substrate specificity as well as diversity of reactions. Some of the P_{450} cytochromes are drug inducible and are presumably involved in the catabolism and detoxification of drug inducer or related compounds. However, other P_{450} cytochromes are present constitutively and little is known about the function of these cytochromes or their specific substrates. Second, we have noted some differences in GS inactivation with phenobarbital induced LM_2 as opposed to the methylcholanthine induced LM_4. Therefore, we propose a systematic study of P_{450} cytochromes to determine whether any might potentially function in the inactivation-degradation process in liver. Levine has recently demonstrated that liver GS is inactivated by ascorbate and it is possible that liver GS level is regulated by this inactivation-degradation process.

The third area of investigation is concerned with the generality of the phenomenon. We plan to survey twenty enzymes for inactivation/modification by one or more of the inactivating systems which have been characterized and if

possible to test differential susceptibility to E. coli or other protease preparations. These studies have already been initiated by Fucci in our laboratory.

The fourth major area of investigation is a miscellaneous category of clarification experiments. For example, studies will be undertaken to clarify some of the mechanisms of enzymatic inactivation of GS. Based on a working model of cyclic reduction-reoxidation of iron, it would be important to determine whether iron (II) supports GS activity and such an observation would provide evidence for the site specific mechanism outlined above. Another experiment which was previously outlined was based on the observation that some P_{450} mediated hydroxylations result from peroxidase activity of P_{450}. In the presence of organic peroxides, the requirement for the electron donor and reducing system is bypassed. It would be interesting to determine if an analogous reaction occurred with respect to GS inactivation. Such a reaction should then be catalase insensitive. Another important experiment is to determine whether GS immunological cross reaction is lost following inactivation in in situ experiments in E. coli and catalase-deficient strains.

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Protein Structure: Enzyme Action and Control

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
Research in this section consists of studies on the physical and chemical properties of proteins of biological interest and the roles of ligand binding and protein-protein interactions in enzyme catalysis and regulation. (1) Active-site ligand interactions with dodecameric glutamine synthetase from E. coli have been studied by spectrophotometric, binding, and calorimetric techniques using the diastereoisomers of L-met-S,R-sulfoximine separately and together. Conformational differences between unadenylylated and adenylylated enzyme forms on binding active-site ligands have been detected. (2) Reactivation of glutamine synthetase after auto-inactivation with L-met-S-sulfoximine, ATP, and Mn^{2+} (or Mg^{2+}) has been accomplished. The reactivation of the enzyme is first order, dependent on the 3rd-4th power of $[H^+]$, and coincides with the release/subunit of 2 Mn^{2+}, L-met-S-sulfoximine-P, and ADP; increasing the pH from ≤ 5 to > 6 produces reinactivation. Inter- and intra-subunit bonding domains are markedly stabilized in the inactive enzyme complex. (3) Equilibrium and kinetic studies of Mn^{2+}-glutamine synthetase interactions (using metal ion-dye chelators and pH indicator dyes) have provided information on the structural and catalytic roles of Mn^{2+}.

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

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

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

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

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

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

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

Major Findings

(1) On the binding of *S*- and *R*-diastereoisomers of the active-site analog *L*-methionine sulfoximine to glutamine synthetase from *E. coli* (Investigator: A. Shrake). Glutamine synthetase, a strictly regulated enzyme in *E. coli*, is a dodecamer with each subunit containing a catalytic site with two essential divalent cation sites (n_1 and n_2) and a tyrosyl residue which is the site of covalent modification by enzymatically catalyzed adenylylation-deadenylylation reactions. It was previously reported (Shrake *et al.*, 1980) that the binding of *L*-methionine-*SR*-sulfoximine (an active-site analog) to catalytic sites promotes different local and different gross structural changes in unadenylylated ($GS_{\overline{0}}$ or $GS_{\overline{1}}$) and fully adenylylated ($GS_{\overline{12}}$) glutamine synthetase. An equimolar mixture of the *S*- and *R*-isomers of *L*-methionine sulfoximine produces tyrosyl residue perturbations on binding to unadenylylated enzyme but not to adenylylated enzyme. UV difference spectral results indicated that the binding of *L*-methionine sulfoximine produces a local burial of one tyrosyl residue per subunit in the case of the unadenylylated manganese enzyme or a local spectral perturbation of covalently bound 5'-adenylate groups in the case of the adenylylated, manganese enzyme. Substrates (*L*-glutamate or *L*-glutamine) promote similar, but smaller, spectral perturbations of glutamine synthetase. Studies with the separated *S*- and *R*-isomers of *L*-methionine sulfoximine (provided by F. C. Wedler, Penn State University) have shown that essentially the same conformational change is promoted by binding the *S*- and *R*-isomers (either separately or together in a mixture) to the enzyme, with the final conformation governed by the adenylylation state of the enzyme. Despite the similarity in the ligand-promoted UV difference spectra with *S*- and *R*-isomers, the *S*-isomer binds ~ 10-fold more tightly than does the *R*-isomer.

Furthermore, only the S-isomer is in the correct configuration to be phosphorylated by the enzyme in the presence of ATP and Me^{2+} (see section 3). Thus, differences between the free energies of binding and the reactivities of the S- and R-isomers arise from origins other than those reflected in spectral perturbations.

In an extension of spectrophotometric studies, the binding of the S-isomer of L-methionine sulfoximine to $MnGS_{\bar{1}}$ and to $Mn{\cdot}ADP{\cdot}GS_{\bar{1}}$ was measured at 24°C and pH 7.25 using ultrafiltration to separate the protein-bound ligand from the free ligand. For measurement of the free S-isomer in binding studies, an assay was developed using a separate incubation at 37°C with an excess of enzyme, ATP, and Mn^{2+} added to the S-isomer in the unknown sample. Under these conditions, the amount of enzyme subunits inactivated was quantitatively equal to the concentration of S-isomer present (see section 3). This assay of the S-isomer was linear in the range of 10-90% inactivation of glutamine synthetase and was not affected by the presence of the R-isomer. The binding of the S-isomer to $MnGS_{\bar{1}}$ at pH 7.2 (25°C) was found to agree quantitatively with spectrophotometric titration data, indicating that UV difference spectra result primarily from local perturbations produced by binding the active-site ligand to the catalytic site of each enzyme subunit. In the case of $MnGS_{\bar{1}}$ interacting with S-isomer, negative cooperativity in binding is observed with a Hill coefficient ($\bar{n}_H$) of 0.7_6 and a half-saturation value $[S]_{0.5}$ of 36 µM. Measurements of the S-isomer binding to the $Mn{\cdot}ADP{\cdot}GS_{\bar{1}}$ complex gave $K_D' = 0.18$ µM with no cooperativity in binding apparent. Thus, nucleotide enhances the apparent affinity of the unadenylylated, manganese enzyme 200-fold. Furthermore, the binding of ADP at the active site (complexed to Mn^{2+} at the subunit n_2 metal ion binding site) makes the enzyme markedly favor the binding of the S-isomer over that of the R-isomer, with the ratio of affinities for S-/R-isomer ~ 340/1 in the presence of ADP and only ~ 10/1 in the absence of nucleotide. With either the $MnGS_{\bar{1}}$ or the $Mn{\cdot}ADP{\cdot}GS_{\bar{1}}$ complex, the stoichiometry of binding L-methionine-S-sulfoximine was determined to be 1 eq per subunit of the dodecamer. This stoichiometry is the same as that obtained previously with an approximately equimolar mixture of S- and R-isomers.

With a commercial mixture of S- and R-isomers of L-methionine sulfoximine, it appears that both isomers bind to $MnGS_{\bar{1}}$ with about the same affinity. Later, it was found that the S-isomer and R-isomer have values of $[S]_{0.5}$ of 36 µM and 380 µM, respectively, whereas an equimolar mixture of S- and R-isomers has $[S]_{0.5} = 61$ µM. Recently, the binding of the S-isomer to $MnGS_{\bar{1}}$ in an equimolar mixture of S- and R-isomers at 50% of the maximum UV spectral perturbation was determined to see if the binding of S-isomer to some subunits enhances the affinity of other subunits of the dodecamer for the R-isomer. It was found that the binding affinities of both the S- and R-isomer for $MnGS_{\bar{1}}$ are enhanced in the mixture; the affinity ratio for S-/R-isomer is ~ 3 in the mixture in contrast to ~ 10 when measured separately. Since S- and R-isomers bind to the same site of each subunit, the affinity enhancements with the mixture of S- and R-isomers occur through subunit interactions.

(2) Binding enthalpies for glutamine synthetase interactions with S- and R-isomers of the active-site analog L-methionine sulfoximine (Investigator: E.G. Gorman). Active-site ligand interactions with dodecameric glutamine synthetase (GS) from E. coli were studied by calorimetry using the resolved diastereoisomers of L-methionine-S,R-sulfoximine which were provided in pure form by F.C. Wedler (Penn State University). Such measurements give thermodynamic parameters for ligand interactions at the active site; differences observed with the S- and R-isomers could relate to the fact that only the S-isomer of L-methionine

sulfoximine is in the correct configuration to become a transition state analog by phosphorylation with ATP (see section 3). The S- and R-isomers bind to the manganese, unadenylylated enzyme ($MnGS_{\overline{1}}$) to the extent of 1 eq/subunit (section 1) and the same stoichiometry is assumed for the fully adenylylated enzyme ($MnGS_{\overline{12}}$). Calorimetric measurements were made at 30°C and pH 7.1 using both Tris/HCl and HEPES/KOH buffers containing 100 mM KCl and 1 mM $MnCl_2$.

Thermal titrations of the manganese, unadenylylated enzyme with the S-isomer, the R-isomer, and an equimolar mixture of S- and R-isomers were performed in the Tris and HEPES buffers in the range of ~ 15- > 90% saturation of the enzyme. Reciprocal plots of observed ΔH values versus total ligand concentrations were linear within experimental error and were extrapolated to ΔH values for full enzyme saturation. A thermal titration of $MnGS_{\overline{1}}$ in HEPES buffer also was performed with the commercially available mixture of the S- and R-isomers and these results were identical to those obtained with the equimolar mixture prepared from the separate isomers. With the fully adenylylated, manganese glutamine synthetase, calorimetric experiments were performed at 91% saturation of the enzyme with the S-isomer, the R-isomer, and the equimolar mixture of S- and R-isomers in Tris and HEPES buffers. These data were corrected to full enzyme saturation assuming linearity of heat with binding. The calorimetric data in the Tris and HEPES buffers (which have heats of protonation of -11 and -4.8 kcal per mol of H^+, respectively) allow calculation of the eq of H^+ either taken up (plus sign) or released (minus sign) for each binding reaction as well as correction of the observed enthalpy of binding for buffer proton effects. Table 1 shows binding enthalpies and proton effects for active-site ligands interacting with $MnGS_{\overline{1}}$ and $MnGS_{\overline{12}}$. Data for the binding of L-glutamine is included in Table 1 for comparison and is from Shrake, A., Powers, D.M., and Ginsburg, A. (1977) Biochemistry 16, 4372-4381.

Table 1. Binding Enthalpies and Proton Effects[a]

Active-site analog or substrate added to enzyme at pH 7.1 (30°C)	$Mn \cdot GS_{\overline{1}}$ ΔH	$Mn \cdot GS_{\overline{1}}$ H^+ eq	$Mn \cdot GS_{\overline{12}}$ ΔH	$Mn \cdot GS_{\overline{12}}$ H^+ eq
L-Met-S-sulfoximine	-12.9	0	-26.6	+1.6
L-Met-R-sulfoximine	-11.7	0	- 5.5	-0.8
1:1 Mixture of S & R isomers	-18.9	+0.7	-23.3	+1.1
L-Glutamine	- 9.7	0	- 9.1	-

[a]All values are given per mole of enzyme subunit for saturation with ligand; ΔH values are in kcal/mol (± 1.2) and are corrected for buffer proton effects; proton values are in eq/mol (± 0.2) of uptake (+) or release (-).

The results in Table 1 given for $MnGS_{\overline{1}}$ indicate that ΔH is the same within experimental error for binding the S-isomer and the R-isomer separately, with a negligible proton effect in either case. In contrast, ΔH for binding the S- and R-isomeric mixture to $MnGS_{\overline{1}}$ is ~ 7 kcal/mol of subunit more exothermic and 0.7 eq of proton uptake is associated with the binding process. These differences between the binding of the active-site analogs separately and together in an equimolar mixture apparently result from subunit interactions, since the stoichi-

ometry of ligand binding is unchanged. The protein difference spectra produced by the S- and R-isomers separately and together in a mixture are the same (section 1). Thus, calorimetric data show significant differences between the binding of the isomers separately and together which are not apparent in protein difference spectra.

For the fully adenylylated enzyme ($MnGS_{\overline{12}}$) striking differences in binding enthalpies and proton effects are observed with the S-isomer, R-isomer, and the mixture of S- and R-isomers (Table 1). Furthermore, these parameters for $MnGS_{\overline{12}}$ differ substantially from those observed with the unadenylylated enzyme ($MnGS_{\overline{1}}$), whereas ΔH for binding L-glutamine is the same with these enzyme forms. It is probable that the differences in binding enthalpies reflect proton effects. For example, if -12 kcal/mol is assumed for the intrinsic enthalpy change for binding either the S- or R-isomer, the average heat of ionization of protein groups changing their extent of ionization in binding reactions with $MnGS_{\overline{12}}$ can be calculated to be ~ 8 or 9 kcal/mol, which is compatible with the heat of ionization of histidine residues. The stoichiometry of the H^+ uptake or release accompanying the binding of the S-isomer or R-isomer to $MnGS_{\overline{12}}$, respectively, indicates that more than one such ionizable protein residue per subunit are involved. Furthermore, if ~ 50% of the S-isomer and ~ 50% of the R-isomer are binding to $MnGS_{\overline{12}}$ in the equimolar mixture, the expected net proton uptake with the isomeric mixture is ~ + 0.4; instead + 1.1 eq of H^+ uptake is observed which suggests that a subunit-interaction proton effect of + 0.7 eq of H^+/mol of subunit occurs with both $MnGS_{\overline{1}}$ and $MnGS_{\overline{12}}$. With the equimolar mixture of S- and R-isomers, binding enthalpies with $MnGS_{\overline{1}}$ and $MnGS_{\overline{12}}$ are about equal to the sum of the intrinsic ΔH of binding plus the fractional heat of a protein group ionization (0.7 X 10-11 kcal/mol of H^+ uptake). Protein groups perturbed through subunit interactions on binding both S- and R-isomers to the dodecameric enzyme therefore may have relatively large heats of protonation or, alternatively, the additional heat has some other origin.

The differences in H^+ uptake for binding the S-isomer to $MnGS_{\overline{1}}$ and $MnGS_{\overline{12}}$ could relate to the differences in the activity-pH profiles of these enzymes forms. Unadenylylated enzyme has a pH optimum of ~ pH 7.7, whereas that for the adenylylated, manganese enzyme is ~ pH 6.6. Whatever their origin, the large proton effects observed in binding the S- and R-isomers of L-methionine sulfoximine to $MnGS_{\overline{12}}$, in contrast to the negligible proton effects observed with $MnGS_{\overline{1}}$, are indications of conformational differences between $MnGS_{\overline{12}}$ and $MnGS_{\overline{1}}$ that may have catalytic significance.

Spectrophotometric and calorimetric titration results with the unadenylylated, manganese enzyme ($MnGS_{\overline{1}}$) are compared in Table 2, where half-saturation values ($[S]_{0.5}$) and Hill coefficients (n_H) were obtained from corresponding Hill plots. The spectrophotometric data were obtained by A. Shrake and reflect local binding effects (section 1). However, the thermal data includes heats from local binding interactions as well as possible heats from other protein structural perturbations produced by ligand binding.

Table 2. Spectrophotometric and Calorimetric Titration Data

Isomer of L-Met sulfoximine added to $MnGS_{\bar{1}}$	Spectrophotometric Titration		Thermal Titration	
	$[S]_{0.5}$ (mM)	n_H	$[S]_{0.5}$ (mM)	n_H
S-isomer	0.054	0.8	0.048	0.9
R-isomer	0.60	0.9	0.55	1.0
1:1 of S:R	0.12	0.8	0.16	0.8

[a] $[S]_{0.5}$ values from spectrophotometric titrations are given for 30°C using the values obtained at 24°C and ΔH values at 30°C.

There is excellent agreement between the thermal and spectrophotometric titration data indicating that the heats, as in the case of spectral perturbations, are principally derived from binding interactions between the protein and ligand.

Binding and calorimetric data for unadenylylated, manganese glutamine synthetase summarized in Table 3.

Table 3. Thermodynamic Parameters at pH 7.1 (30°C)[a]

Binding Reaction	ΔG' kcal/mol	ΔH kcal/mol	ΔS[c] cal/(deg·mol)
$MnGS_{\bar{1}}$ + L-Met-S-sulfoximine	-5.92[b]	-12.9	-23
$MnGS_{\bar{1}}$ + L-Met-R-sulfoximine	-4.50[b]	-11.7	-24
$MnGS_{\bar{1}}$ + 1:1 of S:R L-Met sulfoximine	-5.46[b]	-18.9	-44
$MnGS_{\bar{1}}$ + L-Glutamine[d]	-2.96	- 9.7	-22

[a] Standard state for H^+ activity at $10^{-7.1}$ M.

[b] ΔG' values at 30°C were determined from binding and spectrophotometric half-saturation values at 24°C (section 1) and the measured ΔH values (this section).

[c] Calculated from: $\Delta G' = \Delta H - T\Delta S$

[d] Data from Shrate, A., Powers, D.M., and Ginsburg, A. (1977) Biochemistry 16, 4372.

The affinity of glutamine synthetase for L-methionine-S-sulfoximine is about 100-fold greater than that for L-glutamine. The enthalpy of binding the S- or R-isomer of the active-site analog is about 2 to 3 kcal more exothermic than that for binding L-glutamine, while the degree of ordering indicated by the

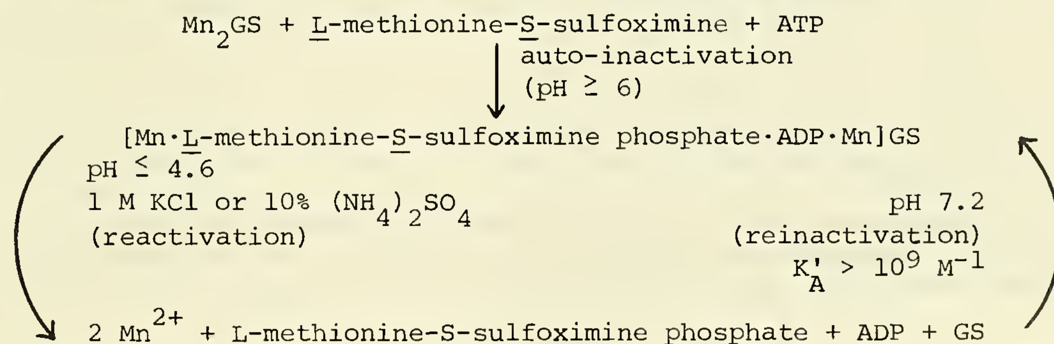
negative ΔS value is about the same for binding either active-site analog or L-glutamine. Since $-T\Delta S = +7$ kcal/mol in these cases, the binding reactions are enthalpically controlled at 30°C. The more negative ΔS calculated for the equimolar mixture of L- and R-isomers could reflect an ordering of water molecules associated with protonation sites.

Studies are in progress on obtaining thermal titration data with $MnGS_I$ and L-glutamate. In addition, we plan to obtain calorimetric data on the interactions of active-site ligands with glutamine synthetase in the presence of nucleotides or nucleotide analogs.

(3) Reactivation of *E. coli* glutamine synthetase after auto-inactivation with L-methionine-S-sulfoximine, ATP, and Mn^{2+} (Investigator: M. R. Maurizi). In the presence of Mn^{2+} (or Mg^{2+}) and ATP, glutamine synthetase catalyzes the phosphorylation of L-methionine-S-sulfoximine. Synergism among the binding of 2 Me^{2+} and the products of the phosphorylation reaction produce very tight binding of all components to the active site of each subunit of the dodecamer, resulting in complete activity loss. The inactive enzyme complex [Mn^{2+}·ADP·L-methionine-S-sulfoximine phosphate·Mn^{2+}]·GS is stable during prolonged dialysis against EDTA (10 mM) and had been disrupted previously only under extreme acidic or alkaline conditions which irreversibly denature the enzyme.

Initial efforts were directed toward determining if any of the components of the inactive enzyme complex were covalently attached to the protein. At neutral pH and room temperature, the complex was considerably resistant to disruption by denaturants such as 8 M urea, 2 M sodium thiocyanate, 2% sodium dodecyl sulfate, or 4.5 M guanidine·HCl. However, incubation of the complex in 6 M guanidine·HCl at pH 7.2 and 25°C for several hours does release Mn^{2+}, ADP, and L-methionine-S-sulfoximine phosphate from the protein. This observation suggests that the components of the complex are not covalently bound since these are not conditions that favor cleavage of covalent bounds.

Further evidence that the ligands are tightly, but reversibly, bound to the enzyme came from the development of conditions which allow dissociation of the enzyme complex and simultaneous recovery of glutamine synthetase catalytic activity. Dissociation of the inactive complex and restoration of enzymatic activity occur at pH < 5 in the presence of high salt concentrations. Reactivation of the enzyme at pH ≤ 5 in the presence of 1 M KCl is a first order process and occurs at the rate of the stoichiometric release of L-methionine-S-sulfoximine phosphate, ADP, or 2 Mn^{2+} from each subunit ($t_{1/2}$ = 2 min at pH 4.1 and 37°C). Neutralization of the solution following reactivation of glutamine synthetase results in the rebinding of the dissociated ligands to the enzyme and consequent reinactivation of the enzyme. These results are summarized in the following scheme:



GS = unadenylylated subunit in dodecameric glutamine synthetase

The reactivation rate increases with decreasing pH (pH 6 to 3) and is proportional to the 3rd or 4th power of [H^+]. Thus, the protonation of 3-4 carboxylate groups per subunit is implicated in the reactivation mechanism. However, merely lowering the pH at ~ 25°C is not sufficient to dissociate the enzyme complex; reactivation also is dependent on the presence of high concentrations of neutral salt and on temperature. At 25°C, KCl, LiCl, NH_4Cl, and NaCl at 1 M concentration considerably accelerate reactivation of the enzyme, whereas 0.5 M K_2SO_4 or $(NH_4)_2SO_4$ are much less effective. Regardless of the salt present, the temperature dependence of reactivation (20 to 60°C) shows an Arrhenius activation energy of ~ 26 kcal/mol. The salt and temperature dependence of reactivation suggests that even at low pH, structural constraints exist within glutamine synthetase which stabilize the complex, possibly by making carboxylate groups less accessible for protonation. In this case, high salt concentrations and temperature increases would produce perturbations of protein structure that are necessary for complex dissociation.

Inactive glutamine synthetase complex produced by auto-inactivation of the enzyme in the presence of Mg^{2+} contains 2 eq of Mg^{2+} tightly bound per subunit (Hunt and Ginsburg, 1980). The inactive complex containing Mg^{2+} is disrupted ~ 5-fold faster than that containing Mn^{2+} under comparable conditions; nevertheless, both forms show the same dependence on pH. The inactive adenylylated, manganese glutamine synthetase complex likewise is reactivated more rapidly than is the unadenylylated, manganese enzyme complex under comparable conditions. The relative stabilities of the inactive glutamine synthetase complexes are consistent with the relative binding affinities the different enzyme forms have for ligands. That is, glutamine synthetase has a higher affinity for Mn^{2+} than for Mg^{2+} and the unadenylylated enzyme has a much higher affinity for Mn^{2+} + ADP than has the adenylylated enzyme.

In studies of the reinactivation reaction shown in the scheme, it was found that the addition of 1 mM EDTA completely blocks reinactivation, that the addition of 1 mM $MnCl_2$ accelerates reinactivation, and that [^{14}C]ADP present originally in the inactive complex was totally exchangeable with added unlabeled ADP (50 μM) after reactivation. Thus, reinactivation requires Me^{2+} and ADP apparently rebinds in this process. With added 1 mM $MnCl_2$, almost total reinactivation of the enzyme was observed within 6 minutes at ~ 25°C with enzyme concentrations as low as 0.2 μM subunits. At pH 7.2 with all dissociated components from the reactivation reaction present, the affinity of the enzyme for any one component is estimated to be > 10^9 M^{-1}.

No evidence of subunit interactions affecting γ-glutamyl transfer activity expression was obtained in studies of the auto-inactivation reaction with unadenylylated, manganese enzyme and the subsequent reactivation reaction. Partial auto-inactivation of manganese glutamine synthetase, performed with substoichiometric amounts of L-methionine-S-sulfoximine or ATP, show that the loss of γ-glutamyl transfer activity is proportional to the amount of the limiting substrate present during auto-inactivation in the range of 10-90% activity loss. (This is the basis of the assay for L-methionine-S-sulfoximine used by A. Shrake in section 1.) Conversely, results from the reactivation reaction show that the γ-glutamyl transfer activity restored is proportional to the amount of complex dissociated in the range of 10-90% reactivation. If it is assumed that the complex forms randomly to subunits of the dodecamer or dissociates randomly from dodecameric inactive glutamine synthetase, then our results indicate that inactive subunits do not affect the γ-glutamyl transfer activity expression of neighboring active subunits.

In the present studies, we have observed some unusual effects of nucleotide which suggest that nucleotide has complex roles in the auto-inactivation and reinactivation reactions shown in the above scheme. As also reported by other workers, the auto-inactivation of glutamine synthetase in the presence of excess substrates is not a first order process, and this has been interpreted by Rhee et al. (1981) as being due to subunit interactions. However, we have seen that the kinetics of the auto-inactivation reaction are greatly dependent on the order of addition of L-methionine-S-sulfoximine and ATP when Mn^{2+} is the divalent cation present or on the ratio of Mg^{2+} to ATP when Mg^{2+} is present. Our results are consistent with a model in which ATP or MeATP binds to and partially stabilizes a form of glutamine synthetase that catalyzes the reaction with L-methionine-S-sulfoximine either slowly or not at all. A rate-limiting interconversion between the resistant and susceptible conformers could explain the nonpseudo-first order kinetics of the auto-inactivation reaction without invoking subunit interactions in such a model. This model is supported also by the observation that the addition of excess ADP (0.5 mM) and Mn^{2+} (1 mM) to the reinactivation mixture containing L-methionine-S-sulfoximine phosphate and ADP protected about 40% of the enzyme subunits present against reinactivation.

Meister and coworkers first demonstrated the auto-inactivation reaction with glutamine synthetases isolated from mammalian sources. Preliminary attempts to reactivate glutamine synthetase in a bovine brain extract were unsuccessful. However, we plan to look more carefully for conditions to reactivate the mammalian enzyme after purifying this octomeric protein from bovine brain. Simultaneously, we will investigate the stoichiometry of Me^{2+} binding to the mammalian enzyme to determine if, as in the case of the E. coli enzyme, 2 Me^{2+} ions per subunit are involved in catalysis.

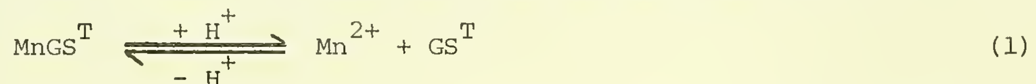
(4) Stabilization of intersubunit bonding domains by active-site ligands binding to glutamine synthetase from E. coli (Investigators: M.R. Maurizi and A. Ginsburg). Dodecameric glutamine synthetase from E. coli dissociates and unfolds very rapidly in 6 M guanidine·HCl. The fully inactive complex of glutamine synthetase containing L-methionine-S-sulfoximine phosphate, ADP, and 2 Mn^{2+} bound per subunit is considerably more stable, requiring 3-4 hours in 6 M guanidine·HCl at pH 7.2 and 37°C for complete dissociation and unfolding. When subunit dissociation and unfolding reactions were measured as a function of time by monitoring decreases in light scattering, the quenching of intrinsic protein tryptophanyl fluorescence, and the increases in the exposure of sulfhydryl groups, these changes coincided with the dissociation of ligands from the inactive enzyme complex. Thus, intersubunit bonding domains are strengthened in the inactive enzyme complex. However, the kinetics of the changes observed with the inactive enzyme complex in 6 M guanidine·HCl were more complex than for a first order process, suggesting that the mechanism of disaggregation and unfolding does not involve simply a rate-limiting dissociation of the complex followed by rapid subunit dissociation and unfolding.

Evidence that stable intermediate oligomeric forms which contain some bound active-site ligands could exist during the disaggregation and unfolding reactions produced by 6 M guanidine·HCl was obtained from the following experiments. After partially inactivating the enzyme to the extent of 20-30% with Mn^{2+}, L-methionine-S-sulfoximine, and ATP, this complex was treated with EDTA and then dithiobisnitrobenzoic acid (DTNB) at pH 8. This treatment caused some subunits without bound inactivating ligands to dissociate from the dodecamer (and to reaggregate into

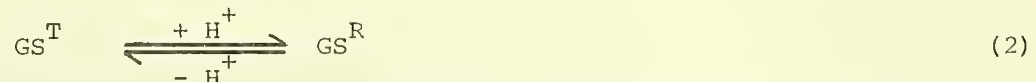
large inactive polymers); undissociated subunits remained as mixed oligomers containing 4,6,8-9, 10-11 active plus inactive subunits. These oligomers were stable during storage at 4°C and could be isolated by HPLC-gel filtration or by polyacrylamide gel electrophoresis under nondenaturing conditions. Preliminary results indicate that the cleavage of DTNB by 2-mercaptoethanol causes reaggregation of the terameric species to octomeric and dodecameric forms. Potentially, an extension of these experiments can give information on the relative strengths of isologous and heterologous subunit bonding domains in glutamine synthetase. Starting with partially inactivated glutamine synthetase, disaggregation intermediates will be further characterized using ultracentrifugal and electron microscopic techniques.

Interactions between active and inactive subunits are responsible for producing stable intermediates during disaggregation of the partially inactivated enzyme. The steps involved in generating the disaggregation intermediates were examined by treating partially inactivated enzyme with 1 mM EDTA, which removes Mn^{2+} (or Mg^{2+}) from n_1 metal ion binding sites and leads to a protein conformational change (relaxation) with fully active glutamine synthetase. Although the rate of relaxation of active subunits was slightly decreased (< 2-fold) upon treatment of the partially inactive enzyme with EDTA at pH 7.2, the active subunits underwent full relaxation. However, less than half of the sulfhydryl groups of the active subunits were modified when the partially inactive enzyme was treated with DTNB at pH 8 after relaxation with EDTA. Preliminary results also indicated that some subunits in disaggregated species can express catalytic activity, but we do not know as yet whether reaggregation occurs under assay conditions.

(5) Glutamine synthetase-Mn^{2+} interactions (Investigators: J.B. Hunt, P.M. Grant, and A. Ginsburg). Studies on the equilibria and kinetics of the binding of Mn^{2+} to, and the release of Mn^{2+} from, glutamine synthetase have continued. Metal ion-dye chelators and pH indicator dyes have been of unique use in these studies. Recent results (Hunt and Ginsburg, 1981) can be described in terms of a two-state model in which glutamine synthetase exists in two conformations: the relaxed enzyme, GS^R, dominates in the absence of divalent cations, and the tightened enzyme, $MnGS^T$, dominates with Mn^{2+} bound to high affinity (n_1) sites. These two enzyme conformations can be distinguished by UV difference spectral measurements. Presently, we are investigating the effects of chelators, substrates, and substrate analogs on the rate constants for Mn^{2+} removal from $MnGS^T$ and Mn^{2+} binding to GS^T and the relaxation reaction. The overall relaxation process involves at least two steps (one fast and the other slow):



$$MnGS^T \underset{-\,H^+}{\overset{+\,H^+}{\rightleftharpoons}} Mn^{2+} + GS^T \quad (1)$$



$$GS^T \underset{-\,H^+}{\overset{+\,H^+}{\rightleftharpoons}} GS^R \quad (2)$$

At 15°C in the absence of effectors, the half-time of the first step is < 1 second, whereas that of the second step is > 2 minutes

Whereas the rate of the conformational change promoted by the binding of Mn^{2+} to n_1 sites of GS^R is not affected by the presence of active-site ligands, the apparent rate of the relaxation reaction 2 is ~ 5-fold slow with L-met-SR-sulfoximine and 2.3-fold slower with L-glutamate than in the absence of effectors

when Mn^{2+} is removed from $MnGS^T$ with EDTA. With or without active-site ligands present, the Arrhenius activation energy was determined to be 30 kcal/mol for reaction 2, which is 9 kcal/mol greater than that of the tightening reaction.

In principle, the active-site ligands could slow down the overall relaxation process either by slowing down the second step or by enhancing substantially the binding of Mn^{2+} to GS^T so as to reduce the instantaneous concentration of GS^T. In an earlier publication (Hunt and Ginsburg, 1980), it was shown that L-met-SR-sulfoximine and L-glutamate enhance the equilibrium binding (K_A') of Mn^{2+} to n_1 sites of the enzyme. Preliminary stopped-flow results indicate that both steps in the relaxation process are slower in the presence of L-met-SR-sufoximine.

In an effort to eliminate any role of EDTA other than that of a divalent cation acceptor in the relaxation process and also to provide solutions of GS^T for direct study of the reaction $GS^T + Mn^{2+} - H^+ \rightarrow MnGS^T$ using a pH indicator dye, we have tried to rapidly remove Mn^{2+} by stirring the $MnGS^T$ complex with Chelex-100 resin for 3 minutes in ice followed by rapid filtration. Because the Arrhenius activation engery for the relaxation reaction is high and the sample can be returned to the temperature of observation in about 30 seconds, good amplitudes are observed for the 290 nm absorbancy change after the chelex treatment. However, the observed half-times were about 20% longer than those for the EDTA-promoted relaxation, and the addition of EDTA after conformational equilibrium produced a further decrease in absorbance at 290 nm from which it is estimated that 5-12% of n_1-site Mn^{2+} remained bound after the Chelex treatment. The rate of relaxation of $MnGS^T$ promoted by 1 mM dipicolinate, which is less efficient than is EDTA in sequestering Mn^{2+}, is about 35% slower than that promoted by 1 mM EDTA although the two chelators eventually produce the same total absorbance decrease at 290 nm. Thus, the availability of a small amount of Mn^{2+} reduces the effective concentration of GS^T, slowing down the apparent rate of conversion to GS^R.

Recently, Ca^{2+} has been used to displace Mn^{2+} at n_1 sites in the presence of xylenol orange (XO). Since the affinity of xylenol orange is much greater for Mn^{2+} than for Ca^{2+}, displaced Mn^{2+} from the protein preferentially binds to the dye to give a large absorbancy increase at 585 or 610 nm. This technique has the advantage that the enzyme with Ca^{2+} bound remains in the tightened conformation. In stopped-flow measurements at pH 7.2 and 15°C, the half-time for the first order rate of Mn^{2+} release from $MnGS^T$ in the reaction $Ca^{2+} + XO + MnGS^T \rightarrow CaGS^T + MnXO$ was in good agreement with the previously measured value for $XO + MnGS^T \rightarrow MnXO + GS^T$. This half-time value (~ 0.25 sec) was increased to 3-6 seconds by the presence of 1 mM L-met-SR-sulfoximine. We can obtain more direct measurements of Mn^{2+} release from $MnGS^T$ using the dye BAPTA [1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-(tetraacetic acid)], described by R.Y. Tsien (Biochemistry 19, 2396-2404, 1980), which binds Ca^{2+}, Mn^{2+}, and Zn^{2+} with high affinity ($\geq 10^7$ M^{-1}). With BAPTA, the half-time value at 15°C for the release of Mn^{2+} from $MnGS^T$ in the presence of L-met-SR-sulfoximine was 4.5 seconds. The dye BAPTA will be used also to determine the rate of release of Ca^{2+} from $CaGS^T$. Through the use of xylenol orange, which has $K_A' = 1.6 \times 10^5$ M^{-1} for Mn^{2+} at pH 7.2 (Hunt and Ginsburg, 1981) and BAPTA, which binds Mn^{2+} stoichiometrically, we will be able to expand our present kinetic studies to include measurements of Mn^{2+} release from the lower affinity n_2 sites as well as Mn^{2+} release from high affinity n_1 sites when active-site analogs are present.

Significance to Biomedical Research

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

Proposed Course of Research

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

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

(3) To characterize the thermal transition of glutamine synthetase more fully in terms of kinetic parameters. Also, the nature of the temperature-induced unfolding reaction (local vs temperature-induced macromolecular shape changes) will be investigated by viscometry, ultracentrifugation, and CD techniques.

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

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

(6) Calmodulin will be purified from bovine brain and thermal titrations of this small modulator protein with Ca^{2+} will be performed. A calorimetric approach, which was developed in measuring ΔH for the substrate-promoted conformational transition of aspartate transcarbamoylase from *E. coli* (Shrake *et al.*, 1981), will be used to determine if Ca^{2+} promotes a conformational change in calmodulin. These studies in conjunction with published binding constants also will allow thermodynamic characterization of Ca^{2+}-calmodulin interactions.

(7) Studies on the removal of Zn^{2+} from aspartate transcarbamoylase (ATcase) from *E. coli* will be performed in collaboration with H. K. Schachman (University of California, Berkeley, California). Since sulfhydryl groups at Zn^{2+} sites on regulatory chains are expected to be highly reactive in the absence of Me^{2+} ions, immediate Me^{2+} ion substitution at Zn^{2+} sites may be necessary to preserve the protein structure. Certain Me^{2+} ions could be useful probes for conformational

changes in ATCase.

Publications

Shrake, A., Whitley, E.J., Jr., and Ginsburg, A.: Conformational differences between unadenylylated and adenylylated glutamine synthetase from Escherichia coli on binding L-methionine sulfoximine. J. Biol. Chem. 255: 581-589, 1980

Hunt, J.B. and Ginsburg, A.: Mn^{2+} and substrate interactions with glutamine synthetase from Escherichia coli. J. Biol. Chem. 255: 590-594, 1980

Whitley, E.J., Jr. and Ginsburg, A.: A novel reaction catalyzed by unadenylylated glutamine synthetase from Escherichia coli: AMP-dependent synthesis of pyrophosphate and L-glutamate from orthophosphate and L-glutamine. J. Biol. Chem. 255: 10663-10670, 1980.

Hunt, J.B. and Ginsburg, A.: Manganese ion interactions with glutamine synthetase from Escherichia coli: Kinetic and equilibrium studies with xylenol orange and pyridine-2,6-dicarboxylic acid. Biochemistry 20: 2226-2233, 1981.

Shrake, A., Ginsburg, A., and Schachman, H.K.: Calorimetric estimate of the enthalpy change for the substrate-promoted conformational transition of aspartate transcarbamoylase from Escherichia coli. J. Biol. Chem. 256: 5005-5015, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00205-26 LB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Role of Selenium in Anaerobic Electron Transport and in Methane Biosynthesis

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

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OTHER:	Gregory Dilworth	Staff Fellow (See individual report)	LB NHLBI
	Wei-Mei Ching	Staff Fellow (See individual report)	LB NHLBI
	Arthur Wittwer	Staff Fellow (See individual report)	LB NHLBI
	Sue H. Neece	Chemist	LB NHLBI
	Joe N. Davis	Chemist (See individual report)	LB NHLBI

COOPERATING UNITS (if any)

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Laboratory of Biochemistry

SECTION

Section on Intermediary Metabolism and Bioenergetics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	1.3	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)

Improved methods of isolation using affinity chromatographic procedures together with compositional studies of the following bacterial selenoenzymes are under investigation: glycine reductase selenoprotein A, Methanococcus vannielii formate dehydrogenase, acetoacetyl-CoA thiolase of Clostridium kluyveri and nicotinic acid hydroxylase of Clostridium barkeri. No selenocysteine residues have been detected in the polypeptide portions of the two latter selenoenzymes. Instead, derivatization procedures result in separation of Se from these proteins in a form that is as yet unidentified. Seleno tRNAs that contain bases specifically modified with selenium have been purified from C. sticklandii and M. vannielii and analyzed. Nuclease digests of tRNAs from both these organisms contain a Se-nucleotide that is less acidic than 4-thiouridylic acid and is thus differentiated from 4-selenouridylic acid.

PHS-6040
(Rev. 2-81)

Project Description

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

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

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

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

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

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

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

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

Major Findings

(1) Continued studies on the clostridial glycine reductase system have concentrated on large scale preparation of enzymes and improved methods of rapid isolation of the selenoprotein A component using antibody columns and diselenide or R-SeH affinity chromatography. Analysis of acid hydrolysates of highly purified selenoprotein to determine the composition of the attached glycosyl groups is carried out by Dr. Sue Neece and J. N. Davis. To date only neutral sugars but no amino sugars have been detected although a variety of hydrolysis conditions have been tried. Linkage of an amino sugar to an N-terminal aspartic acid was thought to afford an explanation of the blocked amino terminus of the protein but if there is no amino sugar present another explanation of the blocked amino terminus will be sought. Glycosyl groups instead could be attached to serine or threonine residues of the protein.

The protein C component of glycine reductase was obtained in about 50% purity earlier and shown to copurify with iron. Highly radioactive enzyme preparations

labeled by growth of Clostridium sticklandii in media containing ^{55}Fe are presently being used to see if this protein actually contains Fe. If so this will facilitate final isolation of the protein in homogeneous form.

(2) In continued work on the selenium-dependent formate dehydrogenase of M. vannielii (in collaboration with Dr. Shigeko Yamazaki, see her individual report) the ^{75}Se-labeled enzyme isolated from bacteria grown with ^{75}Se-selenite has been further characterized as regards subunit structure. In addition to the ^{75}Se-labeled selenoprotein subunit of the enzyme (M_r about 100,000) another ^{75}Se-labeled protein was detected in the M. vannielii extracts. The new selenoprotein exhibited hydrogenase activity indicating yet another naturally-occurring selenoenzyme in bacteria.

As reported last year, a seleno-thiolase (acetoacetyl-CoA thiolase) was discovered in extracts of Clostridium kluyveri and the purification and some properties of this enzyme were determined by M. Hartmanis. To identify the selenium-containing moiety, ^{75}Se-labeled enzyme, purified from extracts of bacteria grown on ^{75}Se-selenite, was reduced with KBH_4 and alkylated with iodoacetamide. This treatment inactivates the enzyme but also results in the cleavage of 35 to 45% of the ^{75}Se from the protein as a low molecular weight component. The cleavage product which was purified by absorption to Dowex-50-H^+ could be differentiated by thin layer chromatography from selenite, Se-alkylated selenocysteine, Se-alkylated selenocysteamine, Selenocystine and selenocystamine. Dissociation of an unidentified selenium compound from the selenothiolase by alkali treatment, as well as by alkaline borohydride reduction, probably explains the earlier failure to detect an ^{75}Se-labeled product in acid hydrolysates of reduced and alkylated ^{75}Se-selenothiolase. Based on present information this selenoenzyme does not appear to contain selenocysteine residues in the polypeptide chains (in contrast to glycine reductase selenoprotein A).

Amino acid transfer nucleic acids (tRNAs) specifically modified with selenium and labeled with ^{75}Se were prepared from extracts of C. sticklandii and M. vannielii. For purposes of seleno base identification, enzymic digestion with nucleases proved to be more satisfactory than mild alkali hydrolysis previously employed. After enzymic digestion of both bacterial tRNA preparations, the oligonucleotide residues were relatively enriched in ^{75}Se suggesting that the selenonucleotide occurs in a sequence somewhat resistant to nuclease attack. However, both tRNA digests contained as the major product a Se-labeled nucleotide that was eluted from DEAE-cellulose after guanylic acid and before 4-thiouridylic acid. This elution pattern in salt gradients serves to differentiate the ^{75}Se-nucleotide from the selenium analog of 4-thiouridylic acid. The latter thionucleotide is present in conspicuous amounts in both C. sticklandii and M. vannielii tRNAs and if selenium incorporation were non-specific it should occur mainly as 4-seleno uridylic acid.

Proposed Course of Research

(1) Using radioactive iron-labeled extracts and newer affinity chromatography procedures experiments are in progress to obtain protein C (Fe protein) of glycine reductase in pure form. Protein B, the carbonyl group protein, is separated in the same procedures and will be stockpiled. Significant amounts of pure selenoprotein A, protein B and protein C are needed for studies on the mechanism of glycine reduc-

tion and identification of the unknown phosphate ester formed in the process.

Studies are continuing to identify the sugars attached in glycosyl linkage to selenoprotein A.

(2) Larger scale studies to identify the selenium moiety of thiolase and the thiolated base(s) of seleno tRNAs will be carried out. ^{75}Se-labeled proteins and tRNAs are used.

(3) In collaboration with S. Yamazaki: establish whether M. vannielii hydrogenase is actually a selenoprotein.

Honors

Secretary and Program Chairman of the American Society of Biological Chemists (three year term ended June 1981).

Member of U.S. National Committee to IUB from Biological Chemistry Division of the American Chemical Society (5 year term).

Elected to membership in the National Academy of Sciences (April 1981).

Invited lecturer at 32nd Mosbach Symposium (Mosbach, Germany) April 23-25, 1981. sponsored by Gesellschaft fur Biologische Chemie.

Publications

T.C. Stadtman. Biological functions of selenium. Trends in Biochem. Sciences (TIBS) 5, 203-206 (1980).

T.C. Stadtman, Gregory L. Dilworth and C.-S. Chen. Selenium Dependent Bacterial Enzymes. Proc. 3rd Int. Symp. on Organic Selenium and Tellurium Compounds. Ed. D. Cagniant and G. Kirsch. Univ. de Metz, pp. 117-130 (1981).

J.B. Jones and T.C. Stadtman. Selenium-dependent and Selenium-independent Formate Dehydrogenases of Methanococcus vannielii. J. Biol. Chem. 256, 656-663 (1981).

S. Yamazaki, L. Tsai, T.C. Stadtman, F.S. Jacobson and C. Walsh. Stereochemical studies of 8-hydroxy-5-deazaflavin-dependent NADP^{+} Reductase from Methanococcus vannielii. J. Biol. Chem. 255, 9025-9027 (1980).

T.C. Stadtman. Bacterial Selenoenzymes and Seleno-tRNAs. Proc. of 2nd Int. Symp. on Selenium in Biology and Medicine. Eds. J. Spallholz and J. Martin. AVI Publishing Co., Westport, CT 1981.

S. Yamazaki, L. Tsai and T.C. Stadtman. Analogues of 8-hydroxy-5-deazaflavin Cofactor: Relative activity as substrates for 8-hydroxy-5-deazaflavin-dependent NADP^{+} Reductase from Methanococcus vannielii. Biochemistry (in press).

T.C. Stadtman. Selenoenzymes. Proc. of 32nd Mosbacher Kolloquium. Gesellschaft fur Biologische Chemie (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00206-22 LB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Stereochemical Studies of Enzymatic Reactions (5-Deazaflavin)

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

P.I.:	Lin Tsai	Reaearch Chemist	LB NHLBI
Other:	S. Yamazaki	Staff Fellow	LB NHLBI

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None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Section on Intermediary Metabolism and Bioenergetics

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.0	0.3

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

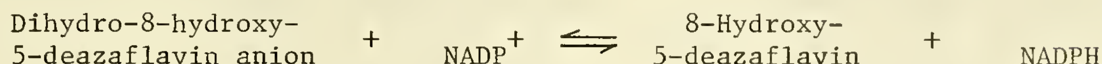
SUMMARY OF WORK (200 words or less - underline keywords)

The chemical and biochemical properties of a number of analogues of 8-hydroxy-5-deazaflavin cofactor were studied and some substrate structure-reactivity relationships between these analogues and 8-hydroxy-5-deazaflavin dependent $NADP^+$ reductase were established. An affinity chromatographic medium for possible use in the purification of 8-hydroxy-5-deazaflavin-dependent enzymes was synthesized.

PHS-6040
(Rev. 2-81)

Project Objectives

8-Hydroxy-5-deazaflavin-dependent $NADP^+$ reductase, a homogeneous enzyme from the extracts of Methanococcus vannielii, catalyzes the reaction:



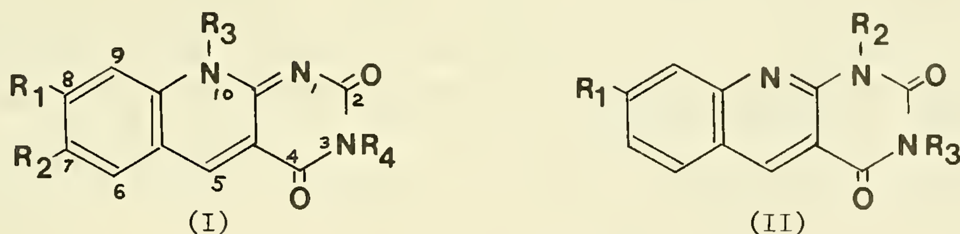
This redox reaction was shown to involve a hydride transfer between the 8-hydroxy-5-deazaflavin cofactor and the pyridine dinucleotide.

(A) In order to establish some substrate structure-reactivity relationships for this enzyme, it is desirable to examine the enzymic reduction of a variety of analogues of the 8-hydroxy-5-deazaflavin cofactor.

(B) The knowledge of the biochemical behaviors of the analogues will serve as the basis for the design and preparation of an affinity chromatographic medium for the purification of 8-hydroxy-5-deazaflavin-dependent enzymes.

Major Findings

(A) The analogues chosen for this study belong to two closely related heterocyclic systems, (I) and (II), where the R's are alkyl, hydroxyl or hydrogen. In order to understand the biochemical behaviors of these analogues, some of the relevant chemical properties were studied.

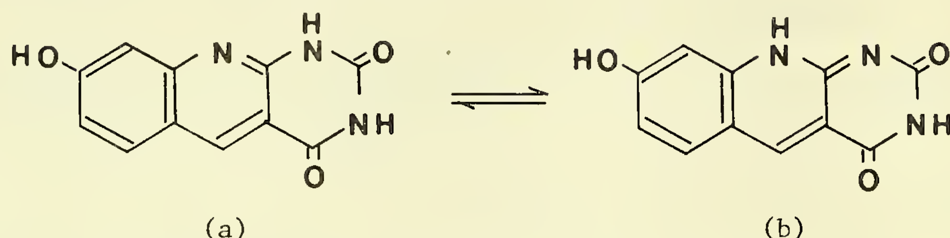


(I) (II)

Although similar in many respects, there are definite distinctions between (I) and (II) as revealed by the differences in their electronic absorption spectra and the pKa's of the 8-hydroxy function; e.g., (I: R_1 = OH, R_3 = Et, R_2 = R_4 = H) pKa = 6.1; (II: R_1 = OH, R_2 = R_3 = H) pKa = 7.2. The more acidic 8-hydroxy function in (I) can be attributed to the greater reasonance stabilization of the anion of (I) than that of (II).

The chemical reduction of (I: R_1 = OH R_3 = Et, R_2 = R_4 = H) by $NaBH_4$ or $NaBH_3CN$ was shown to form a 1,5-dihydro compound on the basis of the ^{1}H-NMR spectrum of the product, which exhibited a new singlet at δ3.45 ppm attributible to the C_5-CH_2 group and the loss of the aromatic C_5-H signal at δ7.31 ppm. The reduction could also be demonstrated in electronic absorption spectrum by the loss of the 415 nm-absorption and the appearance of an absorption band at 320 nm. This observation established the same site of reduction for the chemical as well as the enzymic reaction.

Among the tautomeric states of (II: R_1 = OH, R_2 = R_3 = H), the forms (a) and (b) are of particular interest, because the form (b) belongs to the hetereocyclic

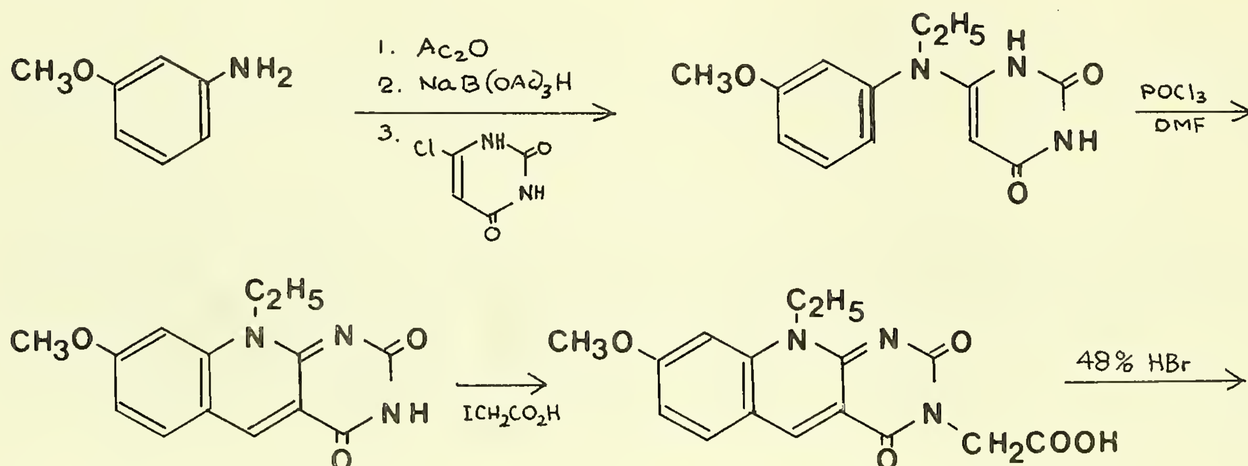


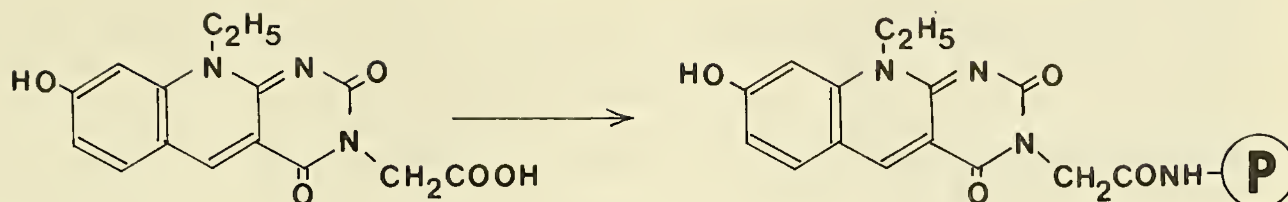
system (I). That the form (a) was the predominent tautomeric state was shown by the similarity of the electronic absorption spectra at neutral pH of (II: R_1 = OH, R_2 = R_3 = H) and (II: R_1 = OH, R_2 = R_3 = CH_3) in which the presence of the N_1-CH_3 group eliminated the possibility of this tautomeric equilibrium.

The kinetic constants for the enzymic reduction of the analogues were determined (Yamazaki). Correlation of the enzymic activity and the structural variations of the analogues suggested the following substrate structure-reactivity relationship:

(i) The minimum structural requirement for the chromophoric group is 10-alkyl (I: R_1 = R_2 = R_4 = H, R_3 = alkyl) or 8-hydroxy (II: R_1 = OH, R_2 = R_3 = H) 2,4-dioxopyrimidino[4,5-b]quinoline, because neither riboflavin nor 1,5-dideazariboflavin undergoes enzymic reduction. (ii) Some steric constraint at C_8 is indicated by the loss of enzymic activity when the 8-hydroxy group is replaced by a methoxy group. (iii) Possible steric constraint at C_7 is shown by the marked decrease in the rate of reduction resulted from the replacement of the C_7-H by CH_3 or OH group. (iv) C_5 is important as the site of reduction since replacement of C_5-H by C_5-CH_3 renders the compound enzymically inactive.

(B) An affinity chromatographic medium for 8-hydroxy-5-deazaflavin-dependent enzyme was synthesized as shown below:





Preliminary experiments (Yamazaki) showed that this affinity chromatographic medium was useful in the purification of a hydrogenase from the extracts of Methanococcus vannielii.

Attempts to prepare another affinity chromatographic medium by the coupling of an analogue of 8-hydroxy-5-deazaflavin cofactor to the polymer through a substituent at the N_{10} position has not yet been successful.

Proposed Course of Action

(1) To continue to study the chemical and biochemical properties of other structural variations of the analogues so as to gain further understanding of the interaction between substrate and enzyme.

(2) To make improvements on the preparation of the affinity chromatographic medium for the 8-hydroxy-5-deazaflavin dependent enzymes.

Publications

S. Yamazaki and L. Tsai. Purification and Properties of 8-Hydroxy-5-deazaflavin-dependent $NADP^+$ Reductase from Methanococcus vannielii. J. Biol. Chem. 255, 6462-6465 (1980).

S. Yamazaki, L. Tsai, T.C. Stadtman, F.S. Jacobson and C. Walsh. Stereochemical studies of 8-hydroxy-5-deazaflavin-dependent $NADP^+$ Reductase from Methanococcus vannielii. J. Biol. Chem. 255, 9025-9027 (1980).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00211-08 LB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Mechanisms of Cellular Regulation - Multiplemolecular Forms of Glutamine Synthetase

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

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	P. Smyrniotis	Research Chemist	LB NHLBI
	M. Wittenberger	Biological Laboratory Technician	LB NHLBI

COOPERATING UNITS (if any)
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SECTION
Section on Enzymes

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Studies were continued on the separation of glutamine synthetase (GS) species that differ in the number (from 0 to 12) and distribution of adenylylated subunits. Some of the difficulties encountered previously in the use of affinity chromatography on Affi-blue sepharose are likely due to (1) variations in the ambient temperature at which the chromatography was performed; (2) heterogeniety in the affinity of Cibacron dye-sepharose complexes that interact with GS; (3) variations in the ratio of ADP to Mn^{2+} that occur during the gradient elutions; and (4) the presence of isomeric forms of GS containing identical numbers of adenylylated subunits. The differential precipitability of GS species containing on the average nearly the same number of adenylylated subunits by means of anti-AMP antibodies is also likely due to the presence of isomeric enzyme forms containing the same number of adenylylated subunits.

PHS-6040
(Rev. 2-81)

Project Description

Previous studies demonstrated that naturally occurring GS preparations are mixtures of hybrid molecules (i.e., molecules containing different proportions of adenylylated subunits) and that partial resolution of these complex mixtures into multiple fractions containing relatively homogenous species of enzyme can be achieved by affinity chromatography on sepharose columns containing either covalently bound Cibacron Blue (Affi-gel blue columns) or covalently bound anti-AMP antibodies (see last year's report). In the present study, these procedures were optimized to obtain greater reproducibility. Also, efforts were made to explain the anomalous behavior of different enzyme preparations when subjected to Affi-gel blue chromatography and to interaction with anti-AMP antibodies.

Major Findings

(1) Affi-blue Sepharose Column Chromatography

The concentration of ADP required to elute GS adsorbed on Affi-blue sepharose increases nearly 1.7-fold as the temperature of chromatography is increased from 30° to 40°C, and 1.8-fold as the concentration of Mn^{2+} is increased from 0.5 mM to 2.0 mM. Addition of 10 mM glutamate to the elution buffer also increases the level of ADP required to elute GS. With continuous linear or exponential gradients, the state of adenylylation of GS (i.e., the number of adenylylated subunits per molecule) eluted increases progressively from 0 to 11 as the concentration of ADP is increased from 0 to 1.5 mM. In contrast, atypical elution profiles are observed if stepwise gradients of ADP are used.

Under all conditions examined, the concentration of ADP required to elute GS with a given state of adenylylation is greater for those GS preparations having a low average state of adenylylation than for those having a high average state of adenylylation. These results and the further observation that anti-AMP antibodies will only precipitate a fraction of GS molecules containing 6-7 adenylylated subunits most likely reflect heterogeniety of GS molecules containing identical numbers of adenylylated subunits.

(2) Binding of Anti-AMP Antibodies to Adenylylated Forms of Glutamine Synthetase

Scatchard plots of binding data obtained when variously adenylylated forms of GS are mixed with increasing concentrations of anti-AMP antibodies yield biphasic curves. Extrapolation of the linear segments of the curves indicate that the antibodies bind with high affinity ($K_d \simeq 1 \times 10^{-7}$ M) to GS with a stoichiometry of one-half antibody molecule per adenylylated subunit, and with low affinity (4.6×10^{-7} M) to GS with a stoichiometry of one antibody molecule per adenylylated subunit. These results suggest that high affinity binding is due either to bivalent interaction of a single antibody with two adenylylated subunits within the same GS molecule (monogamous interaction) or with one adenylylated subunit in each of two different GS molecules (cross-linking interactions). Low affinity binding presumably involves monovalent attachment of a single antibody molecule to each adenylylated subunit on a given GS molecule. These results are in agreement with generally accepted theories for the interactions of multivalent antigens with a bivalent antibody to yield either precipitable aggregates at low antibody/antigen ratios or soluble complexes at high ratios.

The failure to obtain quantitative precipitation of multiply adenylylated forms of GS is therefore likely due to variations in the distribution of adenylylated subunits that favor intermolecular cross-linking reactions on the one hand (precipitable complexes) and monogamous interactions on the other (soluble complexes).

Proposed Course of Action

The above studies will be continued. By means of Affi-gel column chromatography, efforts will be made to isolate large quantities of two different isomeric forms of glutamine synthetase which have the same number of adenylylated subunits. The immunoprecipitability of these isomers by anti-AMP antibodies will be compared and detailed studies of their catalytic activities and their susceptibilities to feedback inhibition by various end products of glutamine metabolism will be examined.

Publications

Hohman, R.J., Rhee, S.G., and Stadtman, E.R.: Anti-AMP antibody precipitation of multiply adenylylated forms of glutamine synthetase from *Escherichia coli*: A model relating both concentration and density of antigenic sites with the antibody-antigen reaction. *Proc. Natl. Acad. Sci.* 77: 7410-7414, 1980.

Meyer, J.M. and Stadtman, E.R.: Glutamine synthetase of pseudomonads: Some biochemical and physiochemical properties. *J. Bacteriol.* 146: 705-712, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00212-10 LB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Regulation of Ammonia-Assimilatory Enzymes in *E. coli* K12

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

P.I.:	Mary Anne Berberich	Research Chemist	LB NHLBI
Others:	Sue Goo Rhee	Research Chemist	LB NHLBI
	Emilio Garcia	Staff Fellow	LB NHLBI

COOPERATING UNITS (if any)

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1.3	1.1	0.2

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

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

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

1. Selection of, and genetic studies on: (a) Mutants of E. coli K12 manifesting alterations in the amount or regulation of the NH_3-assimilatory enzymes (GS, GAT, GDH). (b) Mutants affecting the enzymes and proteins involved in the modification cascade for GS activity.

2. Biochemical characterization of these genetic effects.

3. Physiological studies with otherwise isogenic mutants of class 1 (b) above to determine whether the elements of the modification cascade have an additional regulatory function in nitrogen control.

Major Findings

I. Genetic Studies

(a) Selection of Mutants. Mutants devoid of uridylyltransferase activity ($UTase^-$) have been described previously (Reference 1). Revertants to glutamine independent growth arise spontaneously when MB-1 is plated on nutrient agar in the absence of glutamine. These isolates retained the $UTase^-$ defect and were subsequently shown to be either $ATase^-$ or constitutive GS types ("C"). Phenotypic revertants distributed approximately equally between these two types. In order to facilitate genetic studies, a series of tn5-induced revertants of MB-1 were also isolated. This procedure produced $ATase^-$ and "C" types at a frequency of 3:1.

(b) Mapping Studies. Identity between the tn5 insertion element and the $ATase^-$ or "C" type mutations has not been established, partly due to nonspecific effects of these mutations on sensitivity to kanamycin (tn5 is an insertion element which also carries resistance to kanamycin, an amino glycoside antibiotic). Thorbjornadottir, S.H. et al. (Reference 2) have described several types of spontaneous mutations which cause resistance to kanamycin and related aminoglycosides in E. coli. Two types, unc and ecf, apparently affect the rate-limiting step of accumulation by decreasing the electrochemical gradient or by altering the cell membrane. Other types cause ribosomal alterations. The effects described by these investigators are cumulative and operative within the concentration range used to select for kanamycin resistant insertion mutations. There are indications that selection for kanamycin resistance might simply favor the appearance of $ATase^-$ revertants of MB-1. No transfer of "C" or $ATase^-$ to wild type has been detected using $kanamycin^r$ transfer from plcamch100 donors followed by purification of transductants for KAN^r and CAM^r and assay of isolated recombinants in a whole cell system for GS and $\bar{n}$. $ATase^-$ can be transferred from the putative tn5 insertion mutant via plcam using glutamine-independent growth in the presence of chloramphenicol to identify the transductants. None of these, however, were also KAN^r indicating an absence of linkage between the original tn5 insertion and ATase.

Genetic localization of these mutations is necessary so that they can be removed to $UTase^+$ backgrounds in order to more fully assess their regulatory effects. I am trying to obtain a tn10 transposition element which inserts tetracycline resistance and might prove to be a more useful mutagenic agent in this case. Meanwhile, transduction experiments continue using well characterized recipients and plcam lysates prepared against $ATase^-$ and "C" types as donors. So far, the

data show that ATase⁻ is not linked to glnA or glnD, ilv (unc), pyrE, guaB, and is not carried by the ColE1 plasmid 41-35. It has also been determined that the "C" type mutations are not linked to glnA, glnD, or glnF (argG).

II. Biochemical Characterization of Modification Mutants

Foor, Reuveny and Magasanik (Reference 3) proposed that constitutivity of GS could result from the absence of P_{II} protein. They postulated that P_{II} plays a negative regulatory role in nitrogen control. An extract prepared from an E. coli "C" type was examined for P_{II} protein along with extracts from the wild type parent and ATase$^-$ mutant. The assay depends upon the addition of ATase and UTase purified by Drs. S.G. Rhee and E. Garcia of this laboratory. The deadenylylation of purified GS is followed with time in the presence of Mg^{++}. It was shown that, although both ATase$^-$ and "C" type mutants lack UTase activity, both strains contain P_{II} which functioned as a substrate for the purified UTase added to the reaction mixture. Several ATase$^-$ mutant isolates were also verified in this way. We were able to reexamine the two Klebsiella mutants discussed in Reference 3 and to compare the results with the appropriate parental strain (gifts of Dr. F. Foor) in this partially purified reaction system.

One mutant had been described as possessing an alteration in the P_{II} protein which rendered it a poor substrate for UTase and, consequently, led to low levels of highly adenylylated GS. Another, a tn5-generated deletion mutant, was characterized as devoid of P_{II} protein and thus demonstrated high levels of highly adenylylated GS due, following their hypothesis, to a relief from the negative control imposed by P_{IIA}. All the phenotypic characteristics of these strains were as described when tested in this laboratory. However, it could be demonstrated that extracts from both strains contained P_{II} protein which could function as a substrate for the purified UTase added to the reaction. In addition, it was determined that both mutants were deficient in UTase and that the putative "altered" P_{II} mutant had very little ATase activity. In view of these results and considering the problems encountered with kanamycin resistance and sensitivity, it would appear that this "rare" deletion mutation generated by excision of tn5 and closely linked to guaB requires further biochemical characterization.

III. Physiological Studies

Early in this work (Reference 4), the structural genes for the GS, GAT, and GDH enzyme proteins were located at widely separated sites in the E. coli chromosome. Subsequent studies (Reference 5) indicate that regulation of the synthesis of these proteins occurs via independent genetic sites, although other evidence suggests that activation of the specific effectors involved is probably related in some complex way within the scheme for nitrogen control. For instance, mutations resulting in alteration of the GS modification cascade system affect not only the GS level but also that of GDH and GAT according to several parameters.

Mutations leading to loss of adenylyltransferase activity (ATase$^-$) result in elevated levels of GDH in repressing medium. These mutants ultimately attain only one-half maximal derepression of GS. Other data suggest that the rate of enzyme derepression is also reduced in these ATase$^-$ strains. The "C" type mutations derived from glnD$^-$ are maximally derepressed for GS under all conditions of growth in mineral salts medium and the enzyme is always completely adenylylated. These strains also display elevated levels of GDH. In addition, differences in response to D-glutamate are observed between the otherwise iso-

genic ATase and the constitutive strains.

When 5 mM D-glutamate is added to cells growing in excess NH_3, the specific activities of GS, GDH, GAT can be shown to increase linearly over a 60 minute period. The mechanism of D-glutamate "induction" of these enzymes remains obscure, but the response is specific for D-glutamate among many D-amino acids examined: D-proline, D-arginine, D-methionine, D-histidine, D-alanine, D-valine, D-aspartate, D-glutamine, DL-pyroglutamate, $2CH_3$-glutamate. If the addition is made with equimolar amounts of L-glutamate, this effect cannot be demonstrated. If 10 mM L-glutamine is added with D-glutamate, the increase in specific activity is reduced to 50% of that observed with D-glutamate alone. The increase in specific activity is demonstrable whether D-glutamate is added to permeabilized or unpermeabilized cells even though the process of permeabilization itself increases the specific activity of GS to some extent.

An ascending order of response to D-glutamate addition is observed when the level of GDH is compared in the following strains: $glnA^-$, "C" type, $glnD^-$, $glnF^-$, $glnAo^c$ and $ATase^-$. The $ATase^-$ strain showed a 3.5-fold greater effect than the others. Likewise, the effect of D-glutamate on GS level was greatest in $ATase^-$ strains and not demonstrable in "C" types or the $glnAo^c$ strain. These studies indicate that the D-glutamate effect is independent of GS since glnF mutants demonstrate the GDH response quite well. A decrease in the specific activity of GDH and GAT to 60% expected values is, however, another consequence of the glnF mutation which results in glutamine-dependent growth and eliminates GS antigenic material. It is interesting to note that addition of D-glutamate effects increases in all three of these ammonia assimilatory enzymes, whereas during a 90 minute period immediately following cultural downshift from repressing (glucose-mineral salts-ammonia-glutamine) to derepressing (glucose-mineral salts-glutamine) growth conditions, a linear increase in GS is observed while GDH and GAT remain unchanged. When the concentration of glutamine supplied as sole NH_3 source is 10 mMolar, the increase in $\bar{n}$ parallels a more rapid increase in GS specific activity. An increase in GDH is generally observed in cells grown under the latter condition.

Most recently, attention has been focused on the biphasic growth curve which is observed when cells are grown under derepressing conditions where the concentration of glutamine is 10 mM. All strains except $glnA^-$ and "C" types show an initial period with rapid doubling followed by a period of slower growth in log phase culture. If the ColE1 plasmid 41-35 (carries glnA) is introduced, the biphasic growth can also be observed in the case of $glnA^-$ but not in the case of the "C" type strain whose growth is constant at the slower rate. In addition, strains carrying the glnA plasmid demonstrate a lag of from 45-60 minutes before resuming growth at the slower rate. Examination of extracts prepared from cells harvested during the later, slower growth period did not reveal any expression of the glnA episome in wild type, $ATase^-$, or "C" strains. However, glnA67-ColE1(glnA) showed a 3-fold increase in GS over the expected derepressed level. The expression of the glnA episome during the early, rapid growth phase is currently being examined.

Prusiner *et al.* (Reference 6) found that addition of cAMP to cells growing in excess NH_3 invoked an increase in GS and GDH activities and a decrease in GAT activity. This effect could only be demonstrated in cya^- mutants. If 5 mm cAMP is added to GS partial constitutives growing in repressing medium (*i.e.*, containing NH_3 and glutamine), an increase in GS could be observed following a 60 minute

exposure. Addition of cUMP and cGMP has no effect on the levels of the enzyme. It is interesting to note, however, that the levels of GS, GDH, and GAT are consistently at lowest values for cells growing without added glutamine in a mineral salts-ammonia medium with glycerol as carbon source. These observations would suggest that the role of cAMP in nitrogen regulation is not directly related to its ability to relieve catabolite repression.

Proposed Course of Action

1. Continue with appropriate mutant selection in N99 using tn10.

2. Proceed with genetic analyses of ATase$^-$ and "C" type mutations.

3. Continue to explore the hierarchy of nitrogen control as it pertains to GS, GAT, and GDH.

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

5. Continue with studies on the expression of the multiplicity of the ColEl(glnA) plasmid in *E. coli*.

References

1. Garcia, E., Federici, M.M., Rhee, S.G., and Berberich, M.A.: Glutamine synthetase cascade: Enrichment of uridyltransferase in *E. coli* carrying hybrid ColEl plasmids. Arch. Biochem. Biophys. 203, 181-189, 1980.

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3. Foor, F., Reuveny, Z., and Magasanik, B.: Regulation of the synthesis of glutamine synthetase by the P_{II} protein in *Klebsiella aerogenes*. Proc. Natl. Acad. Sci. 77, 2636-2640, 1980.

4. Berberich, M.A.: Genetic studies in glutamate dehydrogenase, glutamate synthase and glutamine synthetase in *E. coli* K12. In *Enzymes of Glutamine Metabolism*, Academic Press, New York, 1973.

5. Sales, M., Gelber, D.A., and Brenchley, J.E.: Isolation of *S. typhimurium* strains altered in the rel control of glutamate dehydrogenase. Abst. Am. Soc. Microbiol., 1981.

6. Prusiner, S., Miller, R.E., and Valentine, R.C.: Adenosine 3':5' cyclic monophosphate control of the enzymes of glutamine metabolism in *E. coli*. Proc. Natl. Acad. Sci. 10, 2922-2926, 1972.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00217-06 LB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Purine Nucleosides As Biological Effectors

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

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
Purine nucleosides stimulate the phosphorylation of specific proteins in extracts of porcine brain. Inosine and guanosine are most effective. Half maximal stimulation occurs at 15 μM inosine or guanosine. Phosphorylation occurs with either γ-[^{32}P]ATP or [^{32}P]Pi. The phosphorylated proteins have Mr's of 63,000 and 29,000. The properties of the phosphoryl bond suggest it is an acyl linkage. Ca and Mg stimulate the rate of the phosphorylation. Calmodulin is not involved in the reaction. Tissue specificity studies demonstrate that the nucleoside stimulated phosphorylation is found in brain, heart, kidney, testis, but not liver.

PHS-6040
(Rev. 2-81)

Project Description

The long term goals of this research are to further understand some of the control processes in purine metabolism, and to understand the interrelationships of the resulting metabolites with other metabolic pathways and cellular processes. The immediate objective is to elucidate the mechanism by which purine nucleosides (inosine and guanosine) stimulate the phosphorylation of endogenous proteins in cell-free extracts of porcine brain.

Major Findings

Purine nucleosides stimulate specifically the phosphorylation of a unique protein in extracts of porcine brain. Adenosine, inosine, and guanosine are most effective. Uridine and xanthosine both enchanced ^{32}P incorporation about 60% and 30%, respectively, of that observed with inosine or guanosine. Deoxyinosine and deoxyguanosine had only slight activity. Hypoxanthine, cytidine, purine monophosphate nucleotides, and ribose-1-phosphate did not stimulate phosphorylation. The stimulation by adenosine probably involves its prior conversion to inosine since the presence of the adenosine deaminase inhibitor, erythro-9-(2-hydroxy-3-nonyl), adenine hydrochloride (EHNA) prevented the stimulation of phosphorylation by adenosine but not that of inosine. In addition, adenosine does not competitively inhibit the effects of inosine. Half-maximal stimulation occurs at 15 μM inosine or guanosine. The effects of inosine and guanosine are not additive.

The phosphorylated species is likely protein. The phosphoprotein is lost upon treatment with pepsin, not organic solvents or hot trichloracetic acid.

Nucleoside stimulated phosphorylation is observed with γ-[^{32}P]ATP or [^{32}P]Pi. Proteins with the same molecular weight are phosphorylated in each case.

The capacity of nucleosides to stimulate phosphorylation is enhanced by Mg^{2+} and to an even greater extent by Ca^{2+}. Preincubation of the extracts with EDTA leads to a decreased rate of phosphorylation. Calmodulin is probably not involved since phenothiazine which inhibits other calmodulin activated reactions does not inhibit the Ca^{2+} effects on the nucleoside-dependent phosphorylation.

The phosphoryl bond is sensitive to hydroxylamine which is good evidence for an acyl linkage. The bond is very alkali labile; 75% of the inosine stimulated phosphorylation is lost after 20 minutes at pH 8.0. The phosphoryl bond is stable up to 1 hour at pH 2.0.

Experiments were done to determine if nucleoside phosphorylase was involved in the phosphorylation. Formycin B, an effective inhibitor of the reaction, did not diminish the extent of the nucleoside stimulated phosphorylation. The addition of purified nucleoside phosphorylase, albeit calf spleen, did not increase the level of phosphorylation.

The activity was measured in a number of tissues. The activity appears to be highest in porcine cerebrum and cerebellum, but is also present in heart, kidney, and testis. No activity has been demonstrated in liver.

A striking correlation was observed between those nucleosides which are effective in promoting the formation of the phosphorylated intermediate and the change in rate and extent of ATP hydrolysis. It remains to be proven whether nucleosides have a direct effector action on an ATPase system. It is possible

that the hydrolysis of ATP represents a partial reaction which occurs in the absence of other substrates.

Proposed Course of Action

Studies will be focused on:

1. Elucidating the mechanism of the phosphorylation.

2. Determining if this represents an autophosphorylation or an eznyme catalyzed phosphorylation.

3. Examining the ATPase-like activity.

The phosphoproteins and other components of the system will be isolated and characterized.

Relevance to Medicine

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

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00224-04

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Enzyme Mechanism and Regulation

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

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.8	1.5	0.3

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The rate of dissociation of calmodulin from cyclic nucleotide phosphodiesterase is increased as the level of calcium is decreased, indicating complex formation between the enzyme and calmodulin with less than four calcium ions bound. ACTH was found to inhibit the activation of phosphodiesterase by calmodulin by forming a complex with the regulatory protein. Preliminary experiments showed that yeast contains neither calmodulin nor phosphodiesterase activatable by bovine brain calmodulin. (2) The mechanism of activation of proteinase B in the chitin synthetase cascade in yeast was studied. The slow process seems to reflect a slow conformational rearrangement. Activation of proteinase B by SDS was found to be due to increased solubility of the substrate azocoll by the detergent, not due to dissociation of proteinase B inhibitor. (3) With 2-amino-4-phosphorobutyric acid as a deadend competitive inhibitor for glutamine, the kinetic mechanism of the γ-glutamyltransferase reaction of E. coli glutamine synthetase can be shown to be rapid-equilibrium-random. (4) An extension of the theory of continuous variation for the determination of binding stoichiometry is presented. It provides a guide for using the relative magnitudes of protein-ligand complexes at different points to determine the reliability of the data and to calculate the dissociation constant.

PHS-6040
(Rev. 2-81)

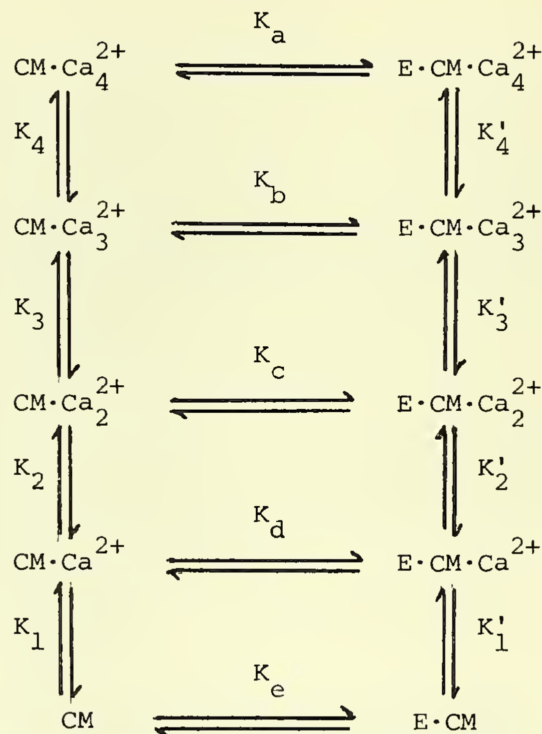
Project Description

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

Major Findings

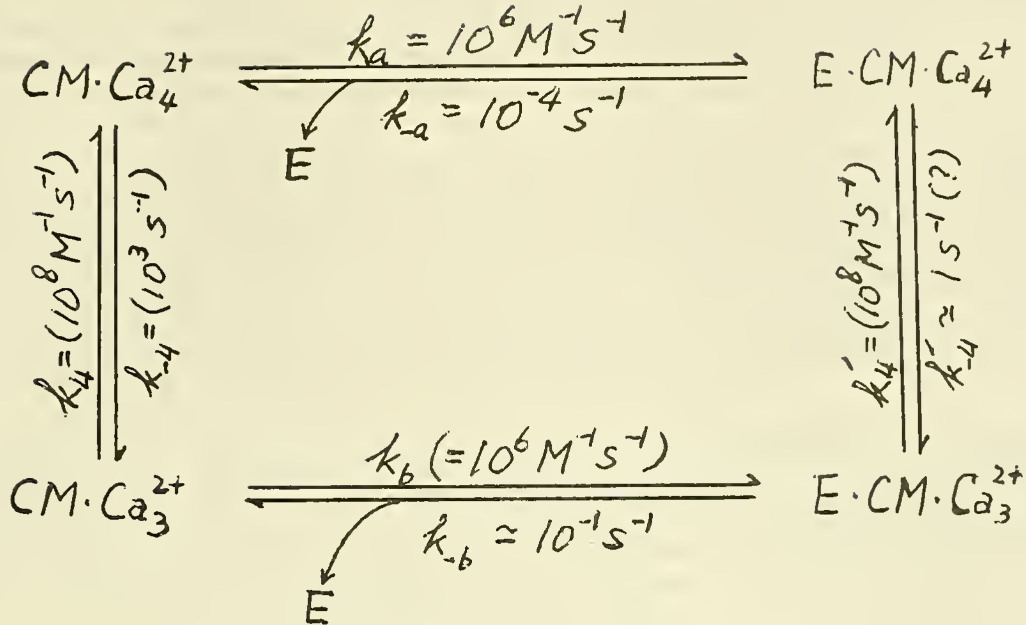
1. Work on the activation, inhibition, and regulation of cyclic nucleotide phosphodiesterase (PDE) has been carried out in collaboration with P. B. Chock and V. Chau. Results not covered in their reports are summarized below:

a. Previous work has established that the activation of PDE requires the binding of Ca^{2+} to all four sites on calmodulin (CM). A scheme which takes into consideration all the interactions involving PDE, CM, and Ca^{2+} has been used to study the system (E = PDE):



Scheme I

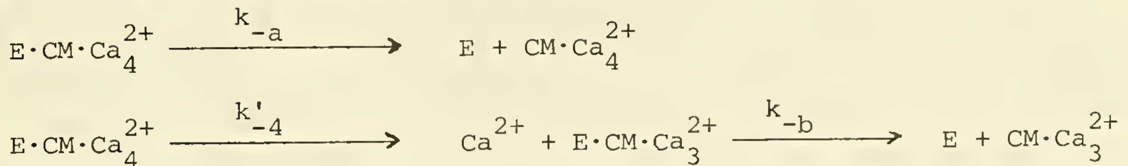
Since the $E{\cdot}CM{\cdot}Ca_4^{2+}$ complex is the sole activated species and the overwhelming dominant species, no information regarding the other enzyme-calmodulin-Ca^{2+} complexes was available from steady-state kinetic studies. An approach utilizing the ability of calmodulin-binding protein (also known as the heat labile inhibitory protein), IP, to compete with PDE for CM was devised to detect the existence of PDE-CM complexes not saturated with Ca^{2+}. Consider the last two steps in Scheme I in terms of rate constants:



Scheme II

The values of k_a and k_{-a} have been experimentally determined and reported in previous reports. k_b is taken to be about the same as k_a, since it is most likely a diffusion-controlled process. k_4 and k_4' are assigned the on-rate constant for Ca^{2+} taken from Eigen's data. The value of k_{-4} is calculated from $K_4 = 10^{-5}$ M determined by our previous Ca^{2+} binding studies. If k_{-4}' is on the order of 1 S^{-1}, then k_{-b} must be on the order of 10^{-1} S^{-1} in order to satisfy the thermodynamic requirement that $K_4K_a = K_bK_4'$. The size of k_{-4}' is a limiting value if one assumes that the affinity of $E\cdot CM\cdot Ca_3^{2+}$ for the fourth Ca^{2+} is 10^3 times higher than that of $CM\cdot Ca_3^{2+}$. $K_4 = 10^{-8}$ M is chosen because such a value makes the $E\cdot CM\cdot Ca_3^{2+}$ complex undetectable under our previous experimental conditions in which the lowest level of Ca^{2+} used was ~ 10^{-7} M.

At subsaturating levels of Ca^{2+}, the fraction of $E\cdot CM\cdot Ca_3^{2+}$ relative to $E\cdot CM\cdot Ca_4^{2+}$ is increased, and the addition of excess IP will permit $CM\cdot Ca_4^{2+}$ to dissociate from PDE by two routes:



$$E\cdot CM\cdot Ca_4^{2+} \xrightarrow{k_{-a}} E + CM\cdot Ca_4^{2+}$$

$$E\cdot CM\cdot Ca_4^{2+} \xrightarrow{k_{-4}'} Ca^{2+} + E\cdot CM\cdot Ca_3^{2+} \xrightarrow{k_{-b}} E + CM\cdot Ca_3^{2+}$$

A faster off-rate for CM is expected if $E\cdot CM\cdot Ca_3^{2+}$ exists at an appreciable level. Other $E\cdot CM\cdot Ca^{2+}$ species are not considered in Scheme II because their contribution should be negligible.

Experimentally, PDE was first allowed to reach maximal activation by the addition of sufficient levels of CM and Ca^{2+} using the continuous, coupled assay at 30°C, pH 8. Small increments of EGTA were then added to reduce the extent of

activation to 30-50%. Excess IP was then added to remove CM and the rate of dissociation of CM was followed as the rate of deactivation of PDE. It was found that the rate of dissociation of CM increased from 10^{-4} S^{-1} to ~ 10^{-3} S^{-1} (estimated from half-time) as the extent of PDE activation was reduced by EGTA from full activation to 30% activation, demonstrating the presence of alternative dissociation pathways and hence the existence of complexes between PDE and CM not saturated with Ca^{2+}. Since IP has been shown to be a Ca^{2+} binding protein, the question may be raised whether the faster dissociation rate is due to further removal of Ca^{2+} by IP. This is unlikely because (i) the assay mixture initially contained 1 mM Ca^{2+}, and the addition of nearly 1 mM EGTA in effect created a buffering system for the Ca^{2+} ions; (ii) removal of Ca^{2+} should cause a sudden drop in PDE activation similar to that observed on addition of EGTA. The experiments will be repeated under conditions where the exact free Ca^{2+} concentrations are known so that the mechanism and rate constants can be accurately analyzed.

b. It has been reported that ACTH and histone H1 inhibit PDE activation by calmodulin. However, it is not clear whether these proteins interact with calmodulin, with PDE, or with the PDE-CM complex. Addition of a μM level of ACTH to CM-activated PDE caused no inhibition, indicating that ACTH does not combine with the PDE-CM complex. However, when EGTA was added to the assay mixture, followed by an equivalent amount of Ca^{2+}, reduction of PDE activity to the basal level was observed. This suggests that ACTH behaves like the calmodulin binding protein. It did not seem to inhibit the PDE-CM complex because of the extremely slow dissociation rate of CM from PDE in the presence of a saturating level of Ca^{2+}. But the stepwise addition of EGTA and Ca^{2+} allowed ACTH to compete with PDE for free CM, hence the rapid inhibition. Complex formation between CM and ACTH has been confirmed by fluorescence studies. Upon adding CM to ACTH, the intrinsic tryptophan fluorescence of ACTH was enhanced by ~ 15%. The high level of ACTH (μM) required to inhibit the action of CM, however, makes a regulatory role for ACTH highly unlikely. ACTH contains many basic amino acid residues, and CM, being an acidic protein, is known to interact with basic proteins. Histone H1 also is a basic protein. It probably inhibits CM activated PDE by a similar mechanism.

c. Calmodulin has been shown to be present in most eukaryotes. In some primitive eukaryotes, the cyclic nucleotide phosphodiesterase has been shown to be activated by calmodulin from mammalian sources, although the presence of indogenous calmodulin could not be demonstrated. In Dr. G. Magni's laboratory at the University of Camerino, Italy, experiments were performed to determine whether calmodulin exists in Baker's yeast and whether yeast PDE can be activated by exogenous calmodulin. Bovine brain calmodulin was partially purified by $(NH_4)_2SO_4$ precipitation, pH 4 precipitation, and heat treatment. Bovine brain PDE was partially purified by $(NH_4)_2SO_4$ precipitation and DEAE-cellulose chromatography. The PDE so prepared is void of calmodulin.

Crude extracts of yeast were prepared from both commercial sources and from newly harvested cells. Preliminary results showed that yeast contains no calmodulin and yeast PDE is not activated by calmodulin. When yeast crude extract was incubated in a boiling water bath for 6 minutes, the resultant supernatant was unable to activate bovine brain PDE. Furthermore, yeast PDE activity remained unchanged upon addition of EGTA. Addition of bovine brain calmodulin and Ca^{2+} also failed to activate yeast PDE. However, these experiments do not rule out the possibility that yeast contains a calmodulin-like protein which does not activate bovine brain PDE. Neither do these experiments rule out the

possibility that yeast PDE is regulated by Ca^{2+} and a calmodulin-like protein, since EGTA has been shown to be without effect on the activity of PDE in the crude extract of adrenal medullus. Upon further purification, however, the effect of EGTA to dissociate the PDE-CM complex from adrenal medullus can be demonstrated.

2. Based upon in vitro experiments, Holzer and coworkers have proposed a cascade mechanism for the activation of chitin synthase in yeast. In this cascade, chitin synthase is activated by limited proteolysis by proteinase B. Proteinase B is activated by proteinase A which degrades proteinase B inhibitor. Proteinase A is, in turn, activated by proteinase B which degrades proteinase A inhibitor. Proteinase A can also be activated by incubation at pH 5. As a result, the activation of proteinase B at pH 5 has been interpreted as the destruction of proteinase B inhibitor by proteinase A.

Elegant as it may appear, this proposed cascade system cannot be sustained by other observations. For example, it has been shown that proteinase B is activated by incubation at pH 5 in the presence of pepstatin, an inhibitor that completely inhibits proteinase A activity. Besides, full activation of proteinase B takes 30-50 hours of incubation at pH 5, 30°C. Such a slow event certainly cannot be considered a viable regulatory process.

To test the hypothesis that a different proteinase may be responsible for the activation of proteinase B at pH 5, the time course of activation of proteinase B at 30°C under five different conditions was followed up to 54 hours. Sample 1 was the control crude yeast extract with no additions. Sample 2 contained 10 mM EDTA to inhibit any metal-requiring proteinases. Sample 3 contained a mixture of metal ions (Co^{2+}, Mn^{2+}, Mg^{2+}, and Ca^{2+}) to detect possible enhancement of activation by metal requiring proteinases. Samples 4 and 5 contained Kunitz inhibitor and a potato inhibitor, respectively. It was found that all five samples yield similar activation curves and no appreciable lag time could be seen during the first 4 hours of activation. In addition, a sample of crude extract which was kept at pH 6.8 at 4°C for 54 hours was found to be nearly fully activated ($> 90\%$). Although it is possible that EDTA, Kunitz inhibitor, or potato inhibitor failed to inhibit the proteinase responsible for proteinase B activation, these results, especially the activation observed at pH 6.8, 4°C, suggest that the slow activation process is likely the manifestation of a slow conformational rearrangement of proteinase B. Previously, it has been reported that proteinase B inhibitor loses its inhibitory effect on incubation with proteinase B at pH 5, while at pH 7, the inhibitory effect of the inhibitor is conserved. It seems that the activation of proteinase B at pH 5 may be explained by its ability to attack the inhibitor at this pH. The present experiments indicate otherwise. First, full activation could be achieved at pH 6.8. Second, a lag phase reflecting the autoactivation process was not seen in the time course of activation of proteinase B.

SDS has been shown to activate proteinase B several folds when the enzyme was assayed with azocoll as the substrate. Consequently, it has been suggested that SDS facilitates the dissociation of inhibitor from proteinase B. This possibility was examined by preincubation of proteinase B with and without 0.2% SDS at pH 6.8, 30°C, for 30 minutes and then assayed with azocoll ± SDS, the final concentration of SDS in the assay was maintained at 0.2%. The time course of assay was also followed at 5 minute intervals up to 30 minutes. It was found that proteinase B activity was completely lost on preincubation with SDS and the

time course of assay with azocoll + SDS was nonlinear, indicating inactivation of proteinase B during assay. Since azocoll is an azotized collagen substrate which is barely soluble in aqueous medium, the activating effect of SDS seems to be due solely to increased solubility of azocoll in the presence of the detergent. In collaboration with Dr. G. Magni, work is in progress to elucidate the actual mechanism of activation of both proteinases A and B.

3. The kinetic mechanism of the γ-glutamyltransferase activity of E. coli glutamine synthetase has been investigated in collaboration with Dr. D. Purich of the University of California at Santa Barbara. With unadenylylated glutamine synthetase in the presence of Mg^{2+}, ADP, and arsenate, at pH 8, the 1/v vs $1/NH_2OH$ (or 1/Gln) plot at various levels of glutamine (or NH_2OH) yields a series of apparently parallel lines. the parallel double reciprocal plots may be interpreted by two different mechanism: (i) a ping-pong mechanism in which glutamine reacts with the enzyme to form a glutamyl enzyme intermediate and NH_3 is released; NH_2OH then reacts with the intermediate to form γ-glutamyl hydroxamate; (ii) a rapid equilibrium-random mechanism in which glutamine and NH_2OH are highly antagonistic to each other's binding (presumably due to competition for the NH_3 site), thereby giving rise to apparently parallel double reciprocal plots. Previous work done with NH_3 as the product inhibitor, hydrazine as the alternative substrate inhibitor, and alanine as the competitive inhibitor showed that the mechanism is random rather than ping-pong. Later it was shown that while alanine behaves like a competitive inhibitor for glutamine in the γ-glutamyltransferase reaction, it does not bind to the glutamine site. For this reason, another compound, 2-amino-4-phosphorobutyric acid (APBA), which has been shown to be a competitive inhibitor for glutamine in the biosynthetic reaction of glutamine synthetase, was used as a kinetic probe. APBA was found to be competitive with glutamine and noncompetitive with NH_2OH. The result confirms that the kinetic mechanism for the γ-glutamyltransferase reaction of glutamine synthetase is rapid-equilibrium-random.

4. In the continuous variation method for the determination of binding stoichiometry, the total molar concentration of protein and ligand is held constant, but their mole fractions are varied. Job (Ann. Chim. (Paris) 9, 113, 1928) first developed the method for the case of all-or-none binding. The theory was later extended by Asmus (Z. Anal. Chem. 183, 321 and 401, 1961) to other cases of one-step binding. The theory can be further extended to stepwise binding for identical and noninteracting sites (Huang, unpublished treatment) using the limiting-slope approach of Asmus. The treatment indicates that the stoichiometry can be accurately determined only if the total molar concentration of protein and ligand is sufficiently large relative to the dissociation constant. Thus, it appears that the method is of limited usage for the following reasons: (i) if the dissociation constant is not known, it must be determined first; (ii) if the dissociation constant is large, the amount of protein required for using the continuous variation method may become unaffordable, and, if the complex formation is to be measured by enzymic activity, the initial velocity may become too difficult to measure. Presented below is a further extension of the theory of continuous variation intended to overcome the limitation and to provide a way of estimating the size of the dissociation constant.

For a protein, P, with n equivalent and noninteracting sites for a ligand, L, we have

$$K_d = \frac{(nP_o - \Sigma)\ (L_o - \Sigma)}{\Sigma} \qquad (1)$$

where P_o = total concentration of P, L_o = total concentration of L, and

Σ = concentration of all forms of P·L complexes. With the continuous variation method, the total protein and ligand molar concentration is held at a constant level C_o such that

$$P_o + L_o = C_o$$

or $$x + y = 1$$

where $$x = P_o/C_o \text{ and } y = L_o/C_o.$$

Consequently, $$P_o = C_o x \text{ and } L_o = C_o (1 - x) \quad (2)$$

Substitution of Equation 2 into Equation 1 yields

$$K_d\Sigma = (nC_o x - \Sigma)(C_o - C_o x - \Sigma) \quad (3)$$

Differentiation of Equation 3 with respect to x results in the following expression:

$$K_d\Sigma' = (nC_o x - \Sigma)(-C_o - \Sigma') + (C_o - C_o x - \Sigma)(nC_o - \Sigma') \quad (4)$$

where $\Sigma' = d\Sigma/dx$:

When $x \to 0$ or $x \to 1$ ($= y \to 0$), $\Sigma \to 0$, and the two limiting slopes are

$$\Sigma'_{x \to o} = nC_o^2/(K_d + C_o) \quad (5)$$

and $$\Sigma'_{y \to o} = nC_o^2/(K_d + nC_o) \quad (6)$$

At the intersection point of the two limiting slopes, we have

$$x_i = (K_d + C_o)/[2K_d + (n + 1) C_o] \quad (7)$$

and $$y_i = (K_d + nC_o)/[2K_d + (n + 1) C_o] \quad (8)$$

where x_i and y_i are the mole fractions x and y corresponding to the intersection point. The number of binding stoichiometry is determined from the ratio of y_i/x_i provided $C_o >> K_d$:

$$\frac{y_i}{x_i} = \frac{K_d + nC_o}{K_d + C_o}$$

$$= n \text{ if } C_o >> K_d$$

The amount of Σ at the intersection point, Σ_i, can be shown from Equations 5 and 7 to be

$$\Sigma_i = x_i\Sigma'_{x \to 0}$$

$$= nC_o^2/[2K_d + (n + 1) C_o] \quad (9)$$

when $n = 1$

$$\Sigma_i = C_o^2/2\ (K_d + C_o) \qquad (10)$$

The mole fraction of P at the apex of the Job curve, x_m, is obtained at $\Sigma' = 0$. Thus, substitution of $\Sigma' = 0$ into Equation 4 yields

$$0 = (nC_o x_m - \Sigma_m)(-C_o) + (C_o - C_o x_m - \Sigma_m)(nC_o)$$

or

$$x_m = [nC_o - (n - 1)\Sigma_m]/2nC_o \qquad (11)$$

where $\Sigma_m = \Sigma$ at the maximum of the Job curve.

Substituting Equation 11 into Equation 3, we have

$$\Sigma_m = n \frac{(n + 1) C_o + 2K_d - 2\sqrt{[(n + 1) C_o + K_d] K_d}}{(n + 1)^2} \qquad (12)$$

when $n = 1$

$$\Sigma_m = \frac{(C_o + K_d) - \sqrt{(2C_o + K_d) K_d}}{2} \qquad (13)$$

Σ_m can be calculated from known k_{cat}- or relative fluorescence, etc., and we can write

$$\Sigma_m = \alpha C_o \qquad (14)$$

where $\alpha = \Sigma_m/C_o$ = amount of Σ at the maximum relative to C_o.

Solving Equations 13 and 14 simultaneously, we get

$$K_d = (2\alpha - 1)^2\ C_o/4\alpha \qquad (15)$$

Thus, K_d can be estimated from the known value of C_o and the experimentally determined value α when $n = 1$. The general equation has the expression

$$K_d = \frac{[(n + 1)\alpha - n]^2\ C_o}{4n\alpha} \qquad (16)$$

Figure 1 illustrates the positions of Σ_i, Σ_m, x_i, and x_m.

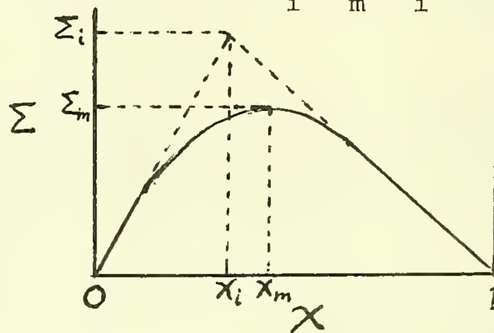


Figure 1

Guidelines for determining n and K_d by the continuous variation method are given below:

(i) Perform experiments for a Job curve and determine the nearest n number by calculating the ratio y_i/x_i, using Table I as a guide for the accuracy of the determination. As a rule of thumb, Σ_m/Σ_i should be around 70%.

(ii) Repeat the experiment by calculating the molar concentration of protein in terms of subunits (or sites), using the n value obtained in (i) to determine the molecular weight of the subunits. A symmetrical Job plot will confirm the n value.

(iii) Calculate K_d from the value of Σ_m and C_o in the symmetrical Job plot according to Equation 15.

It should be noted that Equation 16 is not recommended for the calculation of K_d because the stoichiometry must be confirmed by the symmetrical Job plot (where n = 1). Also, Σ_m, rather than Σ_i, is preferred because the drawing of limiting slopes (for $x \rightarrow 0$ and $y \rightarrow 0$) to obtain Σ_i cannot be accurately done if C_o is not much greater than K_d. Whether the limiting slopes are properly drawn, however, can be checked by the value of Σ_i relative to C_o and the Σ_m/Σ_i ratio (see Table I). For example, if $\Sigma_i = 0.100\ C_o$ and Σ_m/Σ_i is > 70%, the limiting slopes must be in error.

Table I
Expected Values of Σ_i, Σ_m, and Apparent n at Various C_o/K_d Ratios

	n	1	2	3	4	5	6
$C_o = 0.1 K_d$	Σ_i	0.045 C_o	0.087 C_o	0.125 C_o	0.160 C_o	0.192 C_o	0.222 C_o
	Σ_m	0.023 C_o	0.044 C_o	0.063 C_o	0.081 C_o	0.097 C_o	0.113 C_o
	Σ_m/Σ_i (%)	51	51	51	51	51	51
	$n_{apparent}$	1	1.09	1.18	1.27	1.36	1.45
$C_o = K_d$	Σ_i	0.250 C_o	0.400 C_o	0.500 C_o	0.571 C_o	0.625 C_o	0.667 C_o
	Σ_m	0.134 C_o	0.222 C_o	0.286 C_o	0.336 C_o	0.376 C_o	0.409 C_o
	Σ_m/Σ_i (%)	54	56	57	59	60	61
	$n_{apparent}$	1	1.5	2	2.5	3	3.5
$C_o = 5 K_d$	Σ_i	0.417 C_o	0.588 C_o	0.682 C_o	0.741 C_o	0.781 C_o	0.811 C_o
	Σ_m	0.268 C_o	0.400 C_o	0.481 C_o	0.538 C_o	0.580 C_o	0.612 C_o
	Σ_m/Σ_i (%)	64	68	71	73	74	76
	$n_{apparent}$	1	1.83	2.67	3.50	4.33	5.17

Proposed Course of Research

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

2. The role of calmodulin binding protein and its interaction with cyclic AMP and other nucleotides.

3. Relationship between activation and molecular weight change for the calmodulin-cyclic nucleotide phosphodiesterase complexes at various levels of Ca^{2+}.

4. Regulation and activation of proteinases in the chitin synthesis cascade in yeast (in collaboration with Dr. Giulio Magni of the University of Camerino, Camerino, Italy).

5. Kinetic mechanism of uridine phosphorylase from E. coli (also in collaboration with Dr. Giulio Magni).

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Huang, C.Y., and Purich, D.L.: Enzyme activation: Theoretical and experimental aspects. Meth. Enzymol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00225-04 LB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Inactivation of Glutamine Synthetase

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

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

In bacteria, proteolysis of glutamine synthetase is preceded by an oxidative inactivation of the enzyme. This oxidative inactivation is mimicked by a non-enzymic system consisting of ascorbic acid, iron, and oxygen.

Ascorbate-inactivated glutamine synthetase was studied to identify the altered amino acids. Only a single alteration was detected: one of 16 histidine residues was lost. The time course of loss of the residue matches the time course of inactivation.

The ascorbate system may proceed via reduction of ferric to ferrous iron. Ferrous iron could then bind to glutamine synthetase and generate an oxidizing species which destroys the histidine residue.

Such oxidative modifications may provide another mechanism for regulation of enzymic activity, perhaps reversibly. In the case of glutamine synthetase, oxidative inactivation may "mark" the protein for subsequent proteolytic degradation. Oxidative modification may also function in neutrophil-mediated host defense against bacteria.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: Suspensions of cells of *Klebsiella aerogenes* inactivate and then degrade glutamine synthetase. Ascorbic acid also inactivates glutamine synthetase. This reaction provides a model of the inactivation step which occurs within cells. The objectives of this project are determination of the chemical changes which occur on inactivation of glutamine synthetase; purification and characterization of the inactivating and proteolyzing components of the cell; and assessment of the physiologic control of inactivation and proteolysis.

Major Findings

This year's efforts were focused on studies of the chemical changes which occur upon oxidative inactivation of glutamine synthtase and on studies of the oxidizing species.

1. A single histidine residue is lost. Earlier spectral studies suggested that inactivation was accompanied by conversion of a tyrosine residue to a dopa-like compound. Amino acid analyses now show that such is not the case. Only a single residue change was detected: one of the 16 histidine residues/subunit is destroyed in the ascorbate-inactivated enzyme.

This lost histidine might have been a concurrent change and not have been causally related to inactivation. However, study of the time course of inactivation showed that the loss of histidine corresponds precisely to loss of activity. Glutamine synthetase is inactivated by a number of other oxidizing systems, both enzymic and non-enzymic. Drs. L. Fucci and E. Stadtman showed that a diaphorase/NADH system inactivates glutamine synthetase. The system shares many of the characteristics of the ascorbate system. Specifically, the diaphorase-inactivated glutamine synthetase has also lost a single histidine residue.

2. Potential role of ferrous iron. Ascorbate is a reducing agent and is typically thought of as an anti-oxidant. Because iron greatly stimulated ascorbate mediated inactivation of glutamine synthetase, it seemed possible that ascorbate might act by reducing Fe^{+3} to Fe^{+2}. Then the interaction of Fe^{+2} and molecular oxygen could produce an activated oxygen species.

We found that Fe^{+2} plus oxygen does rapidly inactivate glutamine synthetase. (In the absence of oxygen, the enzyme is stable to Fe^{+2}.) Catalase provides partial protection. (Catalase protects completely in the ascorbate system.)

Glutamine synthetase has well characterized divalent binding sites. We speculate that Fe^{+2} binds to such a site and reacts to produce an oxidizing species which destroys a crucial histidine residue.

3. Oxidizing species. If this speculation be correct, the system is an example of a site-directed free radical mechanism. Radicals such as hydroxyl and superoxide as well as singlet oxygen can be potent oxidizing agents. Attempts to implicate one of these three species in the ascorbate-mediated system have been negative thus far: superoxide dismutase does not inhibit the reaction, nor do scavengers of hydroxyl radical such as mannitol, ethanol, and butylated hydroxy-toluene. Singlet oxygen reactions are accelerated 10-fold in D_2O, while the ascorbate inactivation is actually inhibited.

Hypochlorite and H_2O_2 form a potent oxidizing system which may function in neutrophil-mediated killing of bacteria. However, glutamine synthetase is quite

stable to treatment with this system. Also, no I^{125} was incorporated into glutamine synthetase when the ascorbate-mediated inactivation was carried out in the presence of high specific activity I^{125}.

Thus, the actual oxidizing species is unknown.

Significance to Biomedical Research

Studies by C. Oliver, J. Farber, and E. Stadtman in this laboratory demonstrated that oxidatively inactivated glutamine synthetase is more susceptible to proteolytic degradation than is native enzyme. Although the biochemical mechanisms of protein synthesis are known in detail, very little is known of the pathways of protein degradation. Since bacterial cells can catalyze the inactivation and subsequent degradation of glutamine synthetase, we speculate that the oxidative modification of glutamine synthetase may be an initial step in its proteolytic degradation.

Oxidative modification of proteins could be a general phenomenon. Recent reports detail such modifications for a number of proteins, including adenylate cyclase, guanylate cyclase, several lysozomal enzymes, 3-hydroxy-3-methylglutaryl CoA reductase, and α_1-antitrypsin. Some of the oxidatively modified enzymes are inactivated but others show increased activity. Hence, like other covalent modifications, oxidative modification may function physiologically in the regulation of enzymic activity. If so, a reversing, reducing modification will likely occur as well.

Neutrophils produce potent oxidizing agents following phagocytosis. These oxidizing agents are known to alter a number of proteins. In particular, leaking lysozomal enzymes may be oxidatively inactivated to protect against tissue inflammation. These oxidizing agents might also act on bacterial proteins following phagocytosis. For example, oxidative inactivation of bacterial glutamine synthetase could severely disrupt bacterial metabolism and lead to bacterial death.

Proposed Course of Research

1. Studies on the chemical nature of the oxidative modification will continue.

2. Other experiments will examine the generality of oxidative modification. (Purified proteins can be treated in the ascorbate system and then examined for changes in activity or histidine content.)

3. Inactivated glutamine synthetase is proteolytically degraded by extracts of *E. coli*. We plan to purify the components responsible for proteolysis.

Publications

Levine, R.L., Oliver, C.N., and Stadtman, E.R.: Turnover of bacterial glutamine synthetase: Oxidative inactivation precedes proteolysis. *Proc. Natl. Acad. Sci.* 78: 2120-2124, 1981.

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Oliver, C.N., Levine, R.L., and Stadtman, E.R.: Regulation of glutamine synthetase degradation. In (Ed.): Proceedings of the International Symposium on Metabolic Interconversion of Enzymes, Berlin, Springer-Verlag, 1980, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00226-04-LB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Selenoproteins: Synthesis and Role of Selenium

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

P.I.:	Gregory Lee Dilworth	Staff Fellow	LB NHLBI
Other:	Thressa C. Stadtman	Chief, Section on Intermediary Metabolism & Bioenergetics	LB NHLBI

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None

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1.3	1.0	0.3

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Clostridium barkeri incorporates selenium into the enzyme nicotinic acid hydroxylase. Denaturation of the enzyme released the selenium. The selenium moiety can be alkylated when the enzyme is reduced by nicotinic acid. The alkylated selenium product was demonstrated to be dialkyl selenide. The structure of the selenium in the native enzyme is still unknown.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: To elucidate the role of selenium in selenoproteins and to determine the biosynthetic pathway of their synthesis.

Progress

(1) The seleno enzyme nicotinic acid hydroxylase from *C. barkeri* releases selenium upon treatment with denaturing agents (e.g., 1% SDS or 8 M urea) and heat. The released compound can be separated from the bulk of the protein by polyacrylamide gel electrophoresis or gel filtration. Subsequent ion exchange chromatographic procedures show the presence of several selenium-containing compounds. More homogeneous preparations of the selenium-containing moiety were obtained from enzyme reacted with alkylating agents in the presence of the substrate, nicotinic acid.

(2) The selenium compounds released after alkylation had charge properties reflecting the charge properties of the alkylating agents used. Further characterization of these products by comparison to known compounds in a number of chromatographic systems shows that the alkylated product is dialkyl selenide.

(3) The formation of dialkyl selenide upon alkylation of the enzyme indicates the presence of either a selenide complex or an extremely labile selenol or selenoketo compound. Selenocysteine, the selenium moiety found in the three characterized seleno proteins would not be expected to be this labile. Preliminary results are not consistent with the selenium being in the form of an iron-selenium complex analogous to the iron-sulfur complexes found in proteins. The presence of a pterin-like cofactor or pterin-molybdenum cofactor (another potential selenium complex) in nicotinic acid hydroxylase is currently under investigation.

Publications

T.C. Stadtman, Gregory L. Dilworth and C.-S. Chen. Selenium Dependent Bacterial Enzymes. Proc. 3rd Int. Symp. on Organic Selenium and Tellurium Compounds. Ed. D. Cagniant and G. Kirsch. Univ. de Metz, pp. 117-130 (1981).

G. L. Dilworth. Selenium-containing Nicotinic Acid Hydroxylase from *Clostridium barkeri*. Proc. 2nd Int. Symp. on Selenium in Biology and Medicine. Eds. J. Spallholz and J. Martin, AVI Publishing Co., Westport, CT (1981).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00228-04 LB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Purification of An Enzyme of the First Cycle in the Covalent Modification Cascade of Glutamine Synthetase of Escherichia coli

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

P.I.:	Emilio Garcia	Staff Fellow	LB NHLBI
Other:	Sue Goo Rhee	Research Chemist	LB NHLBI
	Earl R. Stadtman	Chief, Laboratory 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 20205

TOTAL MANYEARS: 1.4	PROFESSIONAL: 1.1	OTHER: 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)
We have continued studies on the characterization of the uridylyltransferase (UT) uridylyl-removing enzyme of Escherichia coli. An improved method for the purification of this enzyme has been worked out. We have corroborated the single subunit nature of this bifunctional enzyme by examination of this protein in a filtration column by HPLC. These studies have shown conclusively that the pure and partially purified UT·UR undergoes ready oligomerization to a dimer, trimer, and tetramer form possessing decreased activities. The amino acid composition of UT·UR has been determined.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: The long range objective of this project remains the full *in vitro* characterization of the components of the bicyclic cascade that controls the activity of glutamine synthetase. Using purified UT·UR, we will study some of the kinetic properties of this enzyme, including the role of small molecule effectors such as glutamine, α-ketoglutaric acid, ATP, etc. on its activity as well as on its effects on adenylylation-deadenylylation of glutamine synthetase through its interactions with the other proteins of the cascade.

Major Findings

A. Purification of UT·UR. Several new steps have been tried attempting to optimize a purification scheme previously used. Affinity chromatography on 3-(R_1S)-3-bromo-2-ketoglutarate (an analog of α-ketoglutaric acid known to be a potent activator of UT·UR activities)-linked did not provide any resolution of UTase in several buffer systems. Similarly, the use of chromatofocusing (a recent technique developed by Pharmacia) is unuseful for UT·UR because the low pH required to elute the enzyme causes its inactivation and precipitation in the column.

Two-step new steps were added which increase the efficiency of the purification: a phenyl-sepharose column after the Matrex Blue Gel A column and a final agarose 1.5 sizing column as a last step in the purification. The complete procedures include: (1) use of plasmid-containing strain which overproduces the enzyme, giving a 25-fold effective purification; (2) a polyethylene glycol fractionation; (3) chromatography on DEAE-cellulose; (4) a Matrex Blue Gel A column; (5) a phenyl-sepharose column; and (6) a separation on Agarose 1.5 column. or on Sephacryl-300.

B. Some properties of UT·UR enzyme. Previous studies of pure and partially purified UT·UR by gel filtration and polyacrylamide gel electrophoresis had shown that the enzyme undergoes ready oligomerization to dimer, trimer, and tetramer forms, raising the question as to the true nature of the native form of the enzyme. We have studied this phenomenon by the use of gel filtration by high pressure liquid chromatography and have been able to show conclusively that there is a precursor product relationship between the 100,000 molecular weight monomer and the higher oligomeric forms. If the monomer form is fractionated and collected by this method and reinjected in the HPLC, the whole complement of monomer, dimer, trimer, and tetramer species is obtained. In addition, when the tetramer species is collected from one of these runs and subjected to HPLC in the presence of SDS, a species of identical molecular weight as the monomer is obtained.

We have measured both UT and UR activity of the monomer and oligomeric forms and obtained the two activities at high levels in the monomer and at drastically decreased levels in the oligomers. We do not as yet know if there is a physiological significance for this oligomerization, but it is clear that the two activities reside in the monomer and it is possible that the residual UT and UR activities in the oligomer represents activity contributed by some small population of monomer which is in equilibrium with the oligomer.

We have determined the amino acid composition of the UT·UR enzyme and it is listed below:

Asx 74	ala 67	tyr 22	tvp 11
Thr 30	val 48	Phe 22	pro 76
ser 65	met 6	lys 35	cys 42
gly 96	ile 39	arg 47	
glu 75	leu 91	his 14	

Using the amino acid composition (mole %) and the molecular weight of 92,000-93,000 independently from UT·UR gradient native gels, SDS polyacrylamide electrophoresis, and gel filtration on HPLC, we can arrive at the residues for this enzyme as listed above. An independent determination of the tryptophan to tyrosine molar ratio for this enzyme was carried out by Rodney L. Levine in our laboratory, using a newly developed deconvolution technique from the second derivative of the UV spectrum of the enzyme in the presence of SDS.

Proposed Course of Action

1. Using the purified and partially purified UT·UR enzyme, we will carry out the determination of K_m, V_{max}, the role of effector and other kinetic parameters for both of the activities of this bifunctional enzyme.

2. We will begin systematic studies on stabilization of UT·UR. We have not yet been able to solve the instability problem associated with this enzyme. We believe at present that the instability and the oligomerization of this enzyme may be intimately associated. For this reason, we may continue the work begun using HPLC, as it would provide an easy way to study these phenomena without necessitating laborious assay procedures.

3. We will use some of the inactive but highly purified side products of the stabilization studies to obtain antibodies against the enzyme.

4. We may attempt to carry out some studies on the oligomerization and purification of the enzyme by using radioactively labeled UT·UR, which can be obtained more less readily by using techniques that take advantage of the fact that we have the gene that encodes this enzyme (*glnD*) in a plasmid.

5. Work will be started in a project for subcloning the gene encoding UT·UR in a miniplasmid pKN402 (UHUN *et al.*, *Gene* 6: 91-106, 1979). This plasmid is defective in replication control, and at high temperature it replicates to levels in which up to 400-fold amplification of gene products occur. Success in this attempt will greatly simplify the ongoing studies of this enzyme.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00231-03 LB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Development of a Pure *In vitro* Interconvertible Enzyme Cascade System

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

P.I.:	Emily Shacter Noiman	Biochemist (Guest Worker)	LB NHLBI
Others:	Earl R. Stadtman	Chief, Laboratory of Biochemistry	LB NHLBI
	P. Boon Chock	Chief, Section on Metabolic Regulation	LB NHLBI
	Sue Goo Rhee	Research Chemist	LB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.1	0.2

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
Two enzymes, a cyclic-AMP dependent protein kinase and a phosphoprotein phosphatase, have been purified to near homogeneity from bovine heart. It was discovered that myosin light chains, purified from chicken gizzard can be phosphorylated and dephosphorylated by these two enzymes. The phosphorylation site was characterized and found to be the same as that phosphorylated by myosin light chain kinase. Other characteristics of this phenomenon were studied and were published this year. Attempts to use the myosin light chains as a substrate for the cascade system were plagued by their persistent proteolysis, despite extensive efforts to prevent this. Thus, a new substrate (a peptide) was custom synthesized and has subsequently been used to determine the kinetic constants which help define a cyclic cascade system. Incidentally, some interesting properties of both enzymes have been studied and characterized using this peptide. Meanwhile, a fluorescent peptide was syntheized for use in studies on the kinetic mechanism of protein kinase activation by cAMP. Experiments were carried out with Dr. Vincent Chau which showed the applicability of this peptide to research in this area, and the results have been submitted for publication.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: To synthesize a well defined *in vitro* cascade system which can be used to test the kinetic equations and predictions, which have been developed in this laboratory, describing such intracellular systems. Phosphorylation-dephosphorylation of a peptide substrate has been chosen as a model cascade system.

Major Findings

The nanopeptide, leu-arg-arg-ala-ser-val-ala-gln-leu, can be used as a substrate for both cAMP-dependent protein kinase and phosphoprotein phosphatase. The affects of some activators and inhibitors of the enzymatic reactions, such as divalent metal ions and ATP, have been characterized. The fluorescent peptide, leu-arg-arg-trp-ser-leu-gly, was found to be a good substrate and can be used for fast kinetic analysis of both enzymes.

Proposed Course of Action

A few technical problems have yet to be eliminated before this project on cyclic cascade systems can be completed.

Publications

Noiman, E.S.: Phosphorylation of smooth muscle myosin light chains by cAMP-dependent protein kinase. *J. Biol. Chem.* 255: 11067-11070, 1980.

Wright, D.E. and Noiman, E.S., Chock, P.B., and Chau, V.: Novel fluorometric assay for adenosine 3'-5'-monophosphate-dependent protein kinase and phosphoprotein phosphatase activities. *Proc. Natl. Acad. Sci. U.S.*, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NH 00233-03 LB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Manadione-Dependent p-nitrophenyl-phosphatase of Clostridium sticklandii

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

P.I.:	J. N. Davis	Chemist	LB NHLBI
Other:	T. C. Stadtman	Chief, Section on Intermediary Metabolism & Bioenergetics	LB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

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Section on Intermediary Metabolism and Bioenergetics

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NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.0	0.3

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

In order to find the natural substrate of the quinone dependent phosphatase from Clostridium sticklandii, protein components of the C. sticklandii glycine reductase system and E. coli glutamine synthetase were phosphorylated enzymically and tested as substrates.

^{32}P-labelled cell free extract from Clostridium sticklandii also was analyzed for the natural substrate. Immobilized selenocystamine was prepared as a selective affinity adsorbent of selenoprotein A and other selenium containing ligands.

PHS-6040
(Rev. 2-81)

Project Description

1. To isolate and identify the natural substrate of the bacterial quinone dependent p-nitrophenylphosphatase.

2. Analyze the ability of the purified phosphatase to interact with the glycine reductase system or other phosphorylated proteins that might mimic the natural substrate. If direct interaction with the phòsphatase occurs in the glycine reductase system, it would suggest that the phosphatase normally acts as a phosphate carrier in some cases such as an energy-dependent membrane transport system, a regulatory system involving protein phosphorylation and dephosphorylation, etc. In such a system a protein bound phosphate ester might be required as substrate rather than ATP.

Major Findings

(1) Enzymatic phosphorylation of components in the glycine reductase system was attempted employing crude cell free extracts from Clostridium sticklandii and carrier free ^{32}P. After incubation, aliquots were removed from the reaction mix as a function of time and analyzed for ^{32}P incorporation. Audioradiograms demonstrated the presence of radioactive material dependent on both the addition of ADP and glycine. The two labeled products migrated with R_f values indicating they were ^{32}P-labeled ATP and ADP, both of which were expected. No small molecular weight labeled peptide or similar material was detected.

(2) Glutamine synthetase which had been enzymatically adenylylated and then cleaved by a nuclease to give protein bound O-phosphotyrosine residues was tested as a possible substrate for the menadione dependent phosphatase. Earlier results which showed that the menadione dependent phosphatase can hydrolyze [^{32}P]casein, suggest that peptides containing O-phosphoserine or O-phosphothreonine might be the natural substrate of the phosphatase. The menadione-dependent phosphatase failed to hydrolyze the phosphorylated glutamine synthetase which had the phosphate esterified to tyrosine. However, this protein had been denatured prior to nuclease treatment and this might have rendered it unavailable as a substrate.

(3) Clostridium sticklandii cells were grown in the presence of the usual growth media minus added K_2HPO_4. 2 mci of carrier free ^{32}P was added to the growing cells and allowed to continue growing for ^{32}P incorporation. Sonic extract was prepared and passed over a DEAE 52 anion exchange column. A radioactive fraction was eluated with 100 mM NaCl and desalted by Sephadex G-25. Endogenous phosphatase activity was removed by affinity chromatography. The resulting ^{32}P-labeled fraction was assayed as substrate, but not hydrolyzed by the addition of purified phosphatase.

(4) Selenocystamine was immobilized to form a stable peptide linkage using CH-sepharose 4B which had been esterified with N-hydroxysuccinimide. The selenium containing Sepharose gel was prepared for its selective affinity toward selenium containing ligands. Protein A, one of the components of the glycine reductase system has been shown to be a selenoprotein and such an affinity gel can aid in the rapid purification of this protein from crude extract. Chemical or enzymatic phosphorylation of protein A may serve as a substrate for the phosphatase. Additionally the selenocystamine affinity adsorbent may serve to purify selenium containing tRNA or nucleotides.

Proposed Course of Action

(1) The selenoprotein of the glycine reductase complex considered a likely component of that system that might undergo phosphorylation is still the most reasonable candidate as the natural substrate for the phosphatase. Further attempts will be made to phosphorylate this protein either enzymically or chemically.

(2) The radioactive material developed from incubation of crude cell free extract of Clostridium sticklandii and carrier free ^{32}P will be further analyzed both for its identity and ability to serve as substrate for the phosphatase.

(3) Peptides containing serine and threonine residues will be ^{32}P labeled and tested in order to determine the degree of specificity for the hydrolyzable phosphate ester linkage.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00235-03-LB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Properties of 8-Hydroxy-5-Deazaflavin-Dependent Enzymes from Methanococcus vannielii

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

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

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Biochemistry

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Section on Intermediary Metabolism and Bioenergetics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

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)

The ability of the 8-hydroxy-5-deazaflavin-dependent $NADP^+$ reductase to catalyze the reduction of analogues of the natural cofactor was examined. Comparison of the kinetic values revealed certain substrate structure-reactivity relationships for the enzyme.

Hydrogenase was purified from cell-free extracts of Methanococcus vannielii and some evidence for presence of selenium in hydrogenase was obtained.

PHS-6040
(Rev. 2-81)

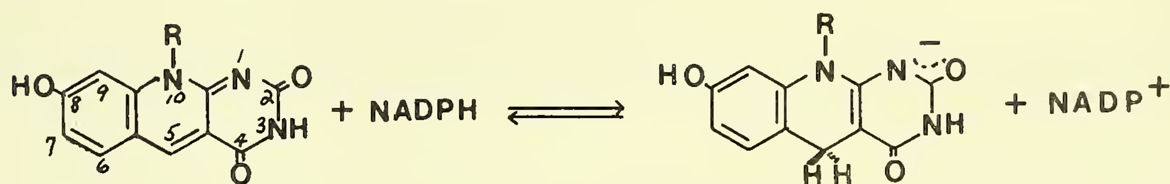
Project Description

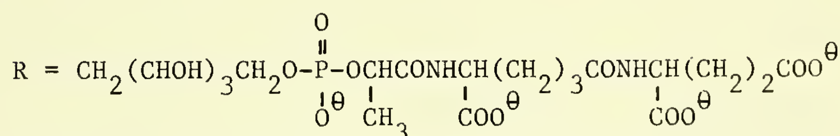
Objectives: The 8-hydroxy-5-deazaflavin cofactor (8-OH-5dFl) that is abundant in methane-producing bacteria serves as electron carrier in coupled enzyme systems, such as a formate-$NADP^+$ reductase system and a hydrogen-$NADP^+$ oxidoreductase system. In Methanococcus vannielii, 8-OH-5dFl, which is reduced by formate (via formate dehydrogenase) or molecular hydrogen (via hydrogenase), serves as cofactor for 8-hydroxy-5-deazaflavin-dependent $NADP^+$ reductase (5-deazaflavin-$NADP^+$ reductase) that reduces $NADP^+$ to NADPH. The 5-deazaflavin-$NADP^+$ reductase specifically requires 8-OH-5dFl as cofactor, whereas both formate dehydrogenase and hydrogenase can utilize a variety of other electron acceptors including flavins and artificial dyes in addition to 8-OH-5dFl.

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

Major Findings

8-Hydroxy-5-deazaflavin-Dependent-$NADP^+$ Reductase. The 5-deazaflavin-$NADP^+$ reductase from M. vannielii catalyzes the reaction





$$R = CH_2(CHOH)_3CH_2O\text{-}\overset{O}{\underset{O^{\ominus}}{P}}\text{-}O\underset{CH_3}{CH}CONH\underset{COO^{\ominus}}{CH}(CH_2)_3CONH\underset{COO^{\ominus}}{CH}(CH_2)_2COO^{\ominus}$$

The ability of the enzyme to catalyze the reduction of analogues of the natural cofactor was examined. Comparison of the kinetic values revealed certain substrate structure-reactivity relationships for the enzyme. Since riboflavin, 1,5-dideazariboflavin and 2,4-dioxopyrimido[4,5-b]quinoline are enzymically inert, the results suggest that the structural requirement of the chromophoric group as an enzyme substrate is 10-alkyl or 8-hydroxy-2,4-dioxopyrimido[4,5-b]quinoline. The study also indicated that there are some steric constraints at C-8, C-7 and C-3 with respect to interaction of the cofactor with the enzyme. Specifically: (a) the 8-methoxy derivative, in contrast to the 8-hydroxy compound, was not reduced, (b) the introduction of a substituent at C-7 or C-3 resulted in a marked decrease in the rate of reduction. The importance of C-5 as the site for the electron entry was suggested by the finding that 5-methyl-5-deazariboflavin was not reduced. The latter inhibited the reduction of 5-deazariboflavin.

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

Recently, two ^{75}Se-labeled proteins were resolved from extracts of M. vannielii cells that had been cultured in the presence of ^{75}Se-selenite by chromatography on a Phenyl-Sepharose column. One of these was the selenium-dependent formate dehydrogenase and the other coincided with hydrogenase activity. The hydrogenase was further purified by sequential chromatography on Phenyl-Sepharose CL-4B and an 8-OH-5dFl affinity column. The purified enzyme showed a single protein band upon native polyacrylamide gel electrophoresis and catalyzed the reduction of tetrazolium dye, 8-OH-5dFl and its synthetic analogues with molecular hydrogen. A ^{75}Se-labeled protein band comigrated with the purified hydrogenase under nondenaturing conditions and its ^{75}Se-labeled subunit appears to have a molecular weight of 45,000 upon SDS gel electrophoresis. Thus, it appears that M. vannielii possesses a selenium-containing hydrogenase in addition to a seleno-formate dehydrogenase. Both of these enzymes are extremely oxygen sensitive.

Proposed Course of Action

(1) Properties of hydrogenase from M. vannielii will be studied.

(2) The molybdenum cofactor of formate dehydrogenase will be isolated and characterized.

(3) Mechanistic and stereochemical studies of 8-OH-5dFl-dependent enzymes will be continued.

Publications

Yamazaki, S. and Tsai, L.: Purification and Properties of 8-Hydroxy-5-deazaflavin-dependent NADP+ Reductase from Methanococcus vannielii. J. Biol. Chem. 255: 6462-6465, 1980.

Yamazaki, S., Tsai, L., Stadtman, T.C., Jacobson, F.S., and Walsh, C.: Stereochemical Studies of 8-Hydroxy-5-deazaflavin-Dependent NADP+ Reductase from Methanococcus vannielii. J. Biol. Chem. 255: 9025-9027, 1980.

Yamazaki, S.: 8-Hydroxy-5-deazaflavin Cofactor Analogues; Relative Activity As Substrates for 8-Hydroxy-5-deazaflavin-Dependent NADP+ Reductase. In Massey, V. (Ed.): Flavins and Flavoproteins, Elsevier, North Holland, 1981, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00237-02 LB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Toxicity and Transport of Bilirubin

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

PI: Rodney L. Levine — Senior Surgeon U.S.P.H.S. (Res.Assoc.) — LB NHLBI

COOPERATING UNITS (if any)
Laboratory of Neurosciences, National Institute on Aging

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.125	0.1	0.025

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Hyperbilirubinemia is probably the most frequent disease which is diagnosed and treated in the human newborn. Current treatment attempts to prevent neurologic damage. Treatment is based on the theory that free (unbound) bilirubin is the toxic fraction of the total bilirubin pool. Proof of this theory is lacking, and certain clinical and experimental findings are not consistent with the theory. Another hypothesis better explains the available data: bilirubin enters the brain bound to albumin when the blood-brain barrier opens. This hypothesis was tested experimentally. We showed that kernicterus does occur when the blood-brain barrier was opened in jaundiced rats. This kernicterus resulted from the entry of albumin-bound bilirubin and not from the passage of free bilirubin. Since the barrier was opened on only one side of the brain, unilateral kernicterus resulted. This provides a convenient animal model of kernicterus, since a control hemisphere is available in the same animal.

PHS-6040
(Rev. 2-81)

Project Description

Introduction

Bilirubin is an end-product of heme metabolism in mammals. *In vitro*, bilirubin is very toxic to cells and to cellular components such as mitochondria. The biochemical basis of the toxicity has not been established.

Hyperbilirubinemia is probably the most commonly diagnosed and treated medical problem of newborn humans. The purpose of treatment is to avoid transport of bilirubin into the brain. If transport and deposition occur, they lead to devastating neurologic damage and the syndrome of kernicterus.

Major Findings

A critical review of neonatal jaundice was undertaken. I found that virtually all medical thinking is based on the premise that free bilirubin (*i.e.*, non-albumin bound) is the toxic fraction of the total pool. Yet no evidence is available to establish this as fact. Further, virtually nothing is known about the mechanism of transport of bilirubin across the blood-brain barrier.

Many experimental and clinical findings appear inconsistent with the free bilirubin theory of transport and toxicity. A new hypothesis was proposed: under certain conditions, the blood-brain barrier opens -- sometimes reversibly. If this occurs in the presence of hyperbilirubinemia, then *albumin-bound* bilirubin will gain access to the brain substance. This hypothesis can explain virtually all of the clinical and experimental characteristics of kernicterus.

The blood-brain barrier can now be reversibly opened in animals by brief infusion of a hypertonic solution into the carotid artery (*Am. J. Physiol.* 238: R421, 1980). The contralateral side is unaffected and serves as a control within the same animal. Permeability returns to normal within hours, and no gross neurologic or behavioral defects have been observed in rats, rabbits, or monkeys.

We used this technique to open the barrier in young adult rats. Then bilirubin was infused peripherally to a peak serum level of about 0.75 mM (40 mg %). Thus, the concentrations of free and albumin-bound species of bilirubin reaching the two cerebral hemispheres were the same. Grossly visible kernicterus occurred in the hemisphere whose barrier was opened, while the control side was spared.

In addition to demonstrating influx of albumin-bound bilirubin, this technique also provides an animal model of kernicterus. Since a control hemisphere is available, studies on the metabolic toxicity of bilirubin should be facilitated.

Significance to Biomedical Research

Current treatment of neonatal hyperbilirubinemia is based on the free bilirubin theory, an unproven concept. Since this may not be the mechanism of transport and toxicity, then treatment programs will require detailed reassessment.

The availability of an animal model of kernicterus will facilitate metabolic and neurophysiologic studies of bilirubin toxicity.

Proposed Course of Research

1. Determine the quantitative relationship between plasma bilirubin clearance and brain bilirubin content.

2. Quantitate the regional differences in bilirubin uptake in the brain, and compare the pattern to that seen in human kernicterus.

3. Study the metabolic effects of bilirubin on cerebral metabolism. Specifically, we plan to apply the 2-deoxyglucose tracer technique.

4. Study possible long term neurologic sequelae, with neuroanatomic correlations.

Publications

Levine, R.L.: Neonatal hyperbilirubinemia. Am. J. Dis. Child. 135: 87, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00239-02 LB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Regulation and Mechanism of Glutamine Synthetase in E. coli

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

P.I.:	Sue Goo Rhee	Research Chemist	LB NHLBI
	Hong Keun Chung	Visiting Fellow (E.O.D. 11/1/80)	LB NHLBI
Others:	Emilio Garcia	Staff Fellow	LB NHLBI
	P. Boon Chock	Chief, Section on Metabolic Regulation	LB NHLBI
	Earl R. Stadtman	Chief, Laboratory of Biochemistry	LB NHLBI

COOPERATING UNITS (if any)
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.3	2.0	0.3

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
(1) Continuation of studies on the bicyclic cascade regulation of glutamine synthetase, involving the development of an improved purification procedure for the uridylyltransferase (UT) and uridylyl-removing (UR) enzyme. Hydrophobic column chromatography on phenyl-sepharose was added. We have shown that a single polypeptide is responsible for both UT and UR activities. HPLC analysis indicated that UT-UR enzyme exists as a mixture of various oligomers and that both UT-UR activities are due to the monomeric form. Molecular weight of monomer evaluated by HPLC was 92,000 ~ 94,000. Amino acid composition of UT-UR was also determined.

(2) Preparation of monoclonal antibodies specific to AMP moiety of adenylylated glutamine synthetase is in progress. BALB/C mice were immunized with BSA-AMP and antibody producing B lymphocytes were fused with myeloma cells.

(3) A photoactive analog of ATP, 8-azido ATP was synthesized. Specific photolabeling of the active site of glutamine synthetase has been attempted without success.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: (1) To purify and characterize UT and UR enzymes from *E. coli*. (2) To establish the catalytic cycle of the biosynthetic reaction catalyzed by adenylylated glutamine synthetase from *E. coli*. (3) To study the interaction between the multideterminant antigen-antibody preparation of monoclonal antibodies specific to AMP moiety of adenylylated glutamine synthetase.

Major Findings

(1) Purification and characterization of a bifunctional protein responsible for uridylyltransferase (UT) and uridylyl-removing (UR) activities (with E. Garcia). Since the activities of UT-UR are very labile, rapid purification of this protein is required. Affinity chromatography on Matrex blue gel A yielded very effective purification. However, the fractions from this column contained very little UT and UR in the presence of high concentration of salts (2 M KCl, 50 mM $MgCl_2$ and 5 mM ATP). Rapid hydrophobic chromatography (less than 2 hours) on phenyl-sepharose permitted further purification of UT-UR with simultaneous removal of salts and 5-10-fold concentration of the protein. This concentrated UT-UR eluted from the phenyl-sepharose column is more than 70% pure. However, when this protein is rechromatographed on the sizing column (Agarose 1.5 M or Sephacryl 300) an extremely broad protein elution profile is obtained, whereas the elution profile for UT-UR activities is much sharper. This result, together with previous observations, indicated that UT-UR undergoes oligomerization which leads to inactivation of both UT and UR activities. This hypothesis was further supported by the HPLC analysis using a Toyo Soda gel filtration column (SW type 3000). When a nearly homogeneous preparation of UT-UR was injected into the HPLC column, at least four peaks (monomer, dimer, tetramer, and aggregate with a higher molecular weight) could be detected under the native condition, while only one major peak was detectable under a denaturing condition (0.1% SDS). Furthermore, reinjection onto the HPLC column of separable protein peaks collected from previous runs with a molecular weight equivalent to either tetrameric or monomeric forms also yielded four peaks. The molecular weight of UT-UR was ~ 93,000 (monomeric form) when measured from native HPLC analysis and ~ 92,000 when measured under denatured conditions.

The amino acid composition of UT-UR (mole %) was determined after acid hydrolysis and the number of individual amino acids listed below were calculated, assuming the molecular weight of UT-UR is 92,000 ~ 93,000. The ratio of tryptophan to tyrosine was obtained by deconvolution technique (developed by Rodney L. Levine in our laboratory) from the second derivative of UV absorbance spectrum measured in the presence of SDS. The number of prolines and cysteines listed below is the calculated value based on the fact that average mole % of proline and cysteine in protein is 2.9% and 5.2%, respectively.

Asx 74	Gly 75	Ile 39	Lys 35	Pro 76
Thr 30	Ala 67	Leu 91	Arg 47	Cys 42
Ser 65	Val 48	Tyr 22	His 14	
Gly 96	Met 6	Phe 22	Trp 11	

Based on the fact that α-ketoglutarate is a potent activator for UT activity, purification of UT-UR was attempted on an affinity chromatography column prepared by linking 3-bromo-ketoglutarate to HS-sepharose (obtained from F. C. Hartman, Oak Ridge National Laboratory). This study was unsuccessful because UT did not

bind to the affinity gel. Another attempt to purify UT-UR involved a new column chromatography technique called "electrofocusing" and this was also unsuccessful, probably because of the low PI of the UT-UR.

(2) Catalytic cycle of the biosynthetic reaction catalyzed by adenylylated glutamine synthetase from E. coli (with G. Ubom and P. B. Chock). The covalently attached AMP moiety of adenylylated glutamine synthetase from E. coli has been replaced by its fluorescent analog, 2-aza-1, N^6-etheno-AMP(aza-ε-AMP). The modified glutamine synthetase (aza-ε-GS) exhibits divalent cation requirement (Mn^{2+}, rather than Mg^{2+}), pH profile, V_{max} and K_m similar to those of naturally adenylylated glutamine synthetase. Whereas naturally adenylylated glutamine synthetase exhibits only negligible fluorescence changes upon the binding of substrates, aza-ε-GS exhibits large fluorescence changes. These fluorescence changes have been used by means of a stopped-flow technique to reveal the involvement of five fluorometrically distinct intermediates in the biosynthetic catalytic cycle of glutamine synthetase. The catalytic cycle is very analogous to that previously established for the unadenylylated enzyme using intrinsic tryptophan fluorescence. Substrates bind randomly, but the reaction proceeds in a stepwise manner. The formation of an enzyme-bound intermediate (probably γ-glutamyl phosphate + ADP) from ATP and L-glutamate is the rate-limiting step, with the subsequent reaction of the enzyme-bound intermediate occurring very rapidly. The success in elucidating this complex mechanism is due largely to the vastly different amplitudes of the fluorescence changes at the two excitation maxima (300 nm and 360 nm) of the aza-ε-AMP moiety which accompany the formation of the various intermediates.

(3) Preparation of hybridomas secreting anti-AMP antibody (with H. K. Chung). Adenylylated glutamine synthetase contains precisely defined antigenic determinants for AMP specific antibody and the epitope density varies as the state of adenylylation changes. To continue the studies on multideterminant antigen-antibody interaction, hybridomas secreting monoclonal antibody specific to AMP was prepared as follows. Adult BALB/c mice were injected intraperitoneally with 0.2 ml of BSA-AMP emulsified in complete Freud's adjuvant. Anti-AMP secreting hybridomas were produced by fusion of spleen cells harvested three days after a booster injection with the 8-azaquanine-resistant NSI myeloma cell line. Cells were distributed into forty-eight culture wells and grown in hypoxanthine/aminopterin/thymidine selective medium. After two weeks, cells were grown in hypoxanthine/thymidine medium and culture supernatants were assayed one week later for anti-AMP activity using adenylylated glutamine synthetase as an antigen. Cloning cells from anti-AMP positive cells is in progress.

(4) Attempted covalent attachment of 8-azido-ADP to the active site of glutamine synthetase. ADP is a nonconsumable substrate for the γ-glutamyltransfer reaction catalyzed by glutamine synthetase. Therefore, if one links ADP properly to its binding site on the glutamine synthetase, the glutamine synthetase catalyzed conversion of glutamine to γ-glutamylhydroxamate will require only NH_2OH and $ASO_4^=$. Glutamine synthetase also converts ATP and methionine-S-sulfoximine (MSOX) to ADP and MSOX-P which are tightly bound to the enzyme. Release of ADP and MSOX-P, as well as recovery of enzyme activity can be achieved only when treated under extreme conditions like incubation at pH 4.0.

Utilizing the fact that ADP binds very tightly in the presence of MSOX-P, it was attempted to photoaffinity-label the active site with azido-ADP formed from azido-ATP and MSOX. Tightly bound azido-ADP and MSOX-P were formed as evidenced by a rapid time-dependent inactivation of glutamine synthetase.

However, irradiation of GS·azido-ADP·MSOX-P complex did not lead to the covalently attached ADP-GS adduct (incubation at pH 4.0 brought the activity of glutamine synthetase back). This experiment was attempted with both Mg·unadenylylated glutamine synthetase and Mn·adenylylated·glutamine synthetase.

Proposed Course of Action

1. Preventing the slow oligomerization seems to be important to stabilizing UT-UR activities. Dissociating reagent-like SDS, urea, guanidine, and sodium borohydride will be tried.

2. Once we learn how to stabilize UR-UT activities, allosteric effectors of various effectors like α-ketoglutarate, glutamine, ATP, Mg and Mn will be studied. The complete bicyclic system will then be investigated.

3. After obtaining monoclonal antibody specific to AMP, monoclonal antibodies specific to a fluorescent analog of AMP, aza-ε-AMP, and specific to unadenylylated glutamine synthetase will be obtained.

Publications

Garcia, E., Federici, M.M., Rhee, S.G., and Berberich, M.A.: Glutamine synthetase cascade: Enrichment of uridylyltransferase in E. coli carrying hybrid ColE1 plasmids. Arch. Biochem. Biophys. 203: 181-189, 1980.

Stadtman, E.R., Hohman, R.J., Davis, J.N., Wittenberger, M., Chock, P.B., and Rhee, S.G.: Subunit interactions of adenylylated glutamine synthetase. In F. Chapeville and A.-L. Haenni (Eds.): Molecular Biology, Biochemistry, and Biophysics, Chemical Recognition in Biology, Vol. 32. Berlin, Springer-Verlag, 1980, 144-156 pp.

Stadtman, E.R., Chock, P.B., and Rhee, S.G.: Regulation of key enzymes by interconversion between covalently modified and unmodified forms. In Smulson and Sugimura (Eds.): Novel ADP-Ribosylations of Regulatory Enzymes and Proteins. New York, Elsevier-North Holland, 1980, 435-448 pp.

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Wedler, F.C., Shreve, D.S., Kenney, R.M., Ashour, A.E., Carfi, J., and Rhee, S.G.: Two glutamine synthetases from Bacillus caldolyticus, An extreme thermophile. J Biol. Chem. 255: 9507-9510, 1980.

Hohman, R.J., Rhee, S.G., and Stadtman, E.R.: Anti-AMP antibody precipitation of multiply adenylylated forms of glutamine synthetase from E. coli: A model relating both concentration and density of antigenic sites with the antibody-antigen interaction. Proc. Natl. Acad. Sci. 77: 7410-7414, 1980.

Rhee, S.G., Chock, P.B., Wedler, F.C., and Sugiyama, Y.: Subunit interaction in unadenylylated glutamine synthetase from E. coli. J. Biol. Chem. 256: 644-648, 1981.

Rhee, S.G., Ubom, G.A., Hunt, J.B., and Chock, P.B.: Fluorometric studies of aza-ε-adenylylated glutamine synthetase from E. coli. J. Biol. Chem. 256: 6010-6016, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00240-02 LB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Purification and characterization of selenium-containing transfer RNAs

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

P.I.:	Wei-Mei Ching	Staff Fellow	LB NHLBI
Other:	Thressa C. Stadtman	Chief, Section on Intermediary Metabolism & 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 20205

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)

Five to 8% of the total tRNA populations from Clostridium sticklandii and Methanococcus vannielii contain selenium modified nucleotides. These Se-nucleotides occur in at least 4 to 6 different tRNA species in both organisms. Two of the C. sticklandii seleno-tRNAs have been isolated in highly purified form. The charging activity of several preparations of one of these (tRNA I) was investigated using both homologous and heterologous (E. coli) a.a.-tRNA synthetases. Although Cys, Glu, Met and Trp seemed to be among the most likely candidates, none of these amino acids was detected in ester linkage to tRNA I.

PHS-6040
(Rev. 2-81)

Project Description

Selenonucleotides have been found to be a natural component of tRNAs in E. coli and certain anaerobic bacteria, e.g. C. sticklandii and M. vannielii. It is presumed that modification of tRNAs with selenium may prove to have some regulatory functions. Current studies have as their aim the purification and identification of these seleno-tRNAs.

Progress

(1) Selenium modified nucleotides occur in at least four different tRNA species in C. sticklandii and five or more in M. vannielii. The amount of selenium found in the tRNAs of both microorganisms is sufficient to modify 5 to 8% of the total population as determined by both atomic absorption and the specific activity of ^{75}Se.

(2) Two of the C. sticklandii seleno-tRNAs (tRNA I and tRNA II) were isolated in highly purified form by a combination of chromatographic techniques, e.g. reversed salt gradient (Sepharose 4B), reversed phase (RPC-5) and ion exchange (DEAE-Sephadex A-50). These two tRNAs exhibit an unusual absorbance at 295 nm and this may be a contribution of the selenobase present in the preparations.

(3) Crude a.a.-tRNA synthetases were isolated from C. sticklandii. When bulk tRNA was incubated with this synthetase preparation in the presence of 15 ^{3}H-labeled amino acids, the incorporation of ^{3}H reached a maximum at 10 minutes and decreased after 3-1/2 hrs. The amount of incorporation could not be increased by adding inorganic pyrophosphatase. Individual a.a.-tRNA synthetase activity was tested using both E. coli tRNA and C. sticklandii tRNA as substrates. In general, most of the synthetases were more active towards E. coli tRNA than C. sticklandii tRNA except for Glu, Met, and Phe.

(4) Highly purified tRNA I failed to be charged by both homologous and heterologous (E. coli) a.a-tRNA synthetases. Although Cys, Glu, Met, and Trp seemed to be the most likely candidates based on observations with less pure preparations. None of these amino acids was detected in ester linkage to tRNA I. This might be due to the instability of the selenonocleotides or the synthetases responsible for charging Se-tRNAs. Unusual lability of the amino acyl bond and/or nonoptimized charging conditions could also be the possibility for uncharging.

Proposed Course of Action

In order to identify the cognate amino acids for Se-tRNAs, two approaches seem applicable:

1) Isolate the Se-tRNA in the acylated form, and determine which amino acids are linked to these Se-tRNAs.

2) Label the tRNAs with radioactive amino acids in vivo and examine the isolated charged tRNAs for the presence of selenium.

After identification, the following studies will be undertaken:

a) Further purify the Se-tRNAs by gel electrophoresis and locate the Se-bases by sequence determination.

b) Study the special functions of Se containing isoacepting tRNAs, the effect of deselenization on its functions, and comparison of non-seleno isoacepting tRNAs regarding charging activities, codon-anticodon recognition, binding to ribosomes and peptide bond formation.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00241-02 LB

PERIOD COVERED
October 1, 1980 to September 30, 1981

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

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

P.I.: Todd Martensen Staff Fellow LB NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.0	0.3

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A methodology for detection and quantitation of protein phosphotyrosine (tyr-P) was developed. The method is rapid (sample preparation and assay ≈ 4 hours) and highly sensitive (detection limit: 100-500 pmol). With ^{32}P labeled proteins, detection can be achieved with trace quantities. The method was used to survey phosphoproteins for tyr-P content. No tyr-P was detected with histone samples and casein. Moreover, when these proteins were phosphorylated with cAMP-dependent protein kinase and casein kinase, no tyr-P was detected. Egg phosphoproteins, phosvitin and vitellin, were shown to contain tyr-P. This is the first identification of phosphotyrosine proteins from nontransformed cells.

Antisera to tyr-P was produced. The IgG fraction which bound tyr-P was purified by affinity chromatography. The phosphotyrosine antibodies showed high affinity for tyr-P and exhibited low cross reactivity with tyrosine, serine phosphate, threonine phosphate, and inorganic phosphate.

A ^{32}P labeled phosphotyrosyl protein was produced for assaying phosphotyrosyl protein phosphatase(s) in cells. The substrate was produced by deadenosylation of [^{32}P]-adenylylated glutamine synthetase. The phosphotyrosyl protein was a substrate for several phosphatases. Detection of phosphotyrosyl protein phosphatase activities in normal and transformed cells is currently being studied.

PHS-6040
(Rev. 2-81)

Project Description

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

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

Major Findings

1. Phosphotyrosine either as a free amino acid or in peptide bonds is sufficiently stable under conditions of rapid base hydrolysis of proteins to allow its detection and quantitation by an amino acid analyzer. Complete hydrolysis of proteins is achieved in 30-35 minutes in 5N KOH at 155°; this results in only 20% loss of tyr-P. The method can quantitate a single residue of tyr-P in 50 µg of a 50,000 molecular weight protein.

2. Phosphotyrosine is not detectable in commercial phosphoprotein samples of calf thymus histones, or in casein. Phosphorylation of histone by cAMP-dependent protein kinase, and casein by casein kinase, did not result in detectable quantities of tyr-P. The phosphoproteins of egg, phosvitin and vitellin, do contain tyr-P. This is the first identification of a protein containing tyr-P in nontransformed cells.

3. A model substrate for the detection and assay of protein phosphotyrosyl phosphatase has been produced by deadenosylation of ^{32}P labeled adenylylated glutamine synthetase by micrococcal nuclease. The phosphotyrosyl which contains a single phosphotyrosine in a defined amino acid sequence is a substrate for bacterial alkaline phosphatase, placental alkaline phosphatase, and calf intestine alkaline phosphatase.

4. Antibodies which bind tyr-P were produced in sheep and purified by affinity chromatography. The purified IgG showed high affinity for tyr-P and low cross reactivity for phosphoserine, phosphothreonine, and tyrosine. The antibody was found to protect phosphotyrosyl glutamine synthetase from digestion with alkaline phosphatase.

Significance to Biomedical Research

The detection and quantitation of tyr-P in proteins by our methodology

should allow researchers to characterize normal and transformed or malignant cells for phosphotyrosine proteins. The role of phosphotyrosyl proteins in cellular activities could then be established. The use of an antibody which bound phosphotyrosyl protein could be most useful in the isolation and, perhaps, histological identification of the cellular location of phosphotyrosyl proteins. The assay of phosphotyrosyl protein phosphatase activity in cells would be important to determine if the regulation of phosphatase activity is important in the turnover of the phosphotyrosyl bond in normal and transformed cells.

Proposed Course of Research

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

Publications

Martensen, T.M.: Phosphotyrosine analysis of proteins and preparation of a substrate for phosphotyrosine phosphatase assay. Fed. Proc. 40: 1539, 1981.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00242-01-LB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

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

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

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

COOPERATING UNITS (if any)

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NHLBI, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Escherichia coli synthesize selenium-containing tRNA when cultured with 0.1 μM $SeO_3^=$ or $SeO_4^=$. The presence of selenium is specific, i.e., the selenium level (about 1 mol Se per 70 mol tRNA) is not affected by the addition of large excesses of sulfur compounds, additional $SeO_3^=$ or $SeO_4^=$, or by a mutation which blocks 4-thiouracil synthesis. The selenium is not present as esterified selenocysteine or selenomethionine.

PHS-6040
(Rev. 2-81)

Project Description

It is known from previous work that *Clostridium sticklandii* and *Methanococcus vannielii* synthesize tRNA which specifically contains selenium, i.e., the level of selenium incorporation is not affected by the addition of 1000-fold excesses of appropriate sulfur compounds.

This project concerns selenium incorporation into the tRNA of *E. coli*. Two initial questions were asked:

1) Do *E. coli* synthesize tRNA which specifically contains selenium?
2) Is the presence of selenium in *E. coli* tRNA due to esterified selenocysteine or selenomethionine?

Major Findings

(1) *E. coli* K-12, when incubated with 0.1 μM $SeO_3^=$, were found to synthesize tRNA which contained 20 to 30 pmol Se per A_{260} unit. This is equivalent to one mol Se per 60 to 80 mol tRNA. This occurred in the presence of 8 mM $SO_4^=$ and was neither decreased by the addition of 1 mM $SO_3^=$, $S^=$, or cysteine; nor increased by the addition of more $SeO_3^=$. Identical Se incorporation was seen from 0.5 μM $SeO_4^=$ in the presence of 8 mM $SO_4^=$, and was not increased by additional $SeO_4^=$.

These observations indicated that selenium was not occurring in tRNA merely as a non-specific replacement for sulfur, otherwise, shifts in the culture medium concentrations of Se- and S-containing compounds would have affected the selenium level in tRNA.

The specificity of selenium incorporation was confirmed by two additional observations. First, when tRNA species synthesized in the presence of $^{35}SO_4^=$ and $^{75}SeO_4^=$ were chromatographically separated, vastly different patterns of S-35 and Se-75 labeling were obtained. Second, an *E. coli* mutant lacking the ability to synthesize 4-thiouracil (the major thiobase in *E. coli* tRNA) synthesized normal levels of selenium-containing tRNA).

(2) The selenium was not separated from the tRNA by incubation at pH 9.0, but could be released by treatment with KBH_4. This suggested that the selenium was present as a base modification and not as esterified selenocysteine or selenomethionine. Amino acid analysis, following reduction and alkylation of the tRNA with KBH_4 and iodoacetate, failed to reveal any trace amounts of carboxymethyl selenocysteine or selenomethionine.

Proposed Course of Action

(1) Investigate the enzymology and cofactor requirements for selenium incorporation into tRNA.

(2) Investigate the chemical nature of the selenium in tRNA.

Publications

Wittwer, A.J. and Wagner, C.: Identification of folate binding protein of mitochondria as dimethylglycine dehydrogenase. (1980) *Proc. Natl. Acad. USA 77*, 4484-4488.

Wittwer, A.J. and Wagner, C.: Identification of the folate-binding proteins of rat liver mitochondria as dimethylglycine dehydrogenase and sarcosine dehydrogenase. Purification and folate-binding characteristics (1981) J. Biol. Chem. 256, 4102-4108.

Wittwer, A.J. and Wagner, C.: Identification of the folate-binding proteins of rat liver mitochondria as dimethylglycine dehydrogenase and sarcosine dehydrogenase. Flavoprotein nature and enzymatic properties of the purified proteins (1981) J. Biol. Chem. 256, 4109-4115.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00243-01 LB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Analysis of An Inactivated Form of the Glutamine Synthetase of *E. coli*

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

P.I.:	Joshua Farber	Staff Fellow (Res.Assoc.)	LB NHLBI
Other:	Rodney L. Levine	Senior Surgeon, USPHS (Res.Assoc.)	LB NHLBI

COOPERATING UNITS (if any)
None

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Laboratory of Biochemistry

SECTION
Section on Enzymes

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NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.4	1.1	0.3

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Previous work in this laboratory has shown that during bacterial growth conditions under which glutamine synthetase activity is being lost, antigenically cross-reacting material initially persists, suggesting that inactivation precedes degradation. Examination of the inactivating activity of cell-free extracts led to the development of a model inactivating system including only ascorbic acid, iron, and oxygen as necessary components. Amino acid analysis revealed the loss of one histidine residue per subunit in the inactivated species. Further analysis was attempted exploiting the ability of the protease subtilisin to clip the glutamine synthetase subunit in two. It was discovered that the inactivated glutamine synthetase was more susceptible to proteolysis than the native enzyme, both in the presence and absence of substrate (ammonium sulfate). It was shown in the case of the proteolysis of the native enzyme that the loss of intact subunit precisely paralleled the attendant loss of enzymatic activity, suggesting that the cleavage of the susceptible peptide bond in this protein could be followed by enzymatic assay. In so doing, it was determined that in this interaction, the K_m of subtilisin for glutamine synthetase subunit is ~ 0.8 μmolar.

PHS-6040
(Rev. 2-81)

Project Description

Previous work in this laboratory has shown that during bacterial growth conditions under which glutamine synthetase (GS) activity is being lost, antigenically cross-reacting material initially persists, suggesting that inactivation precedes degradation. Examination of the inactivating activity of cell free extracts led to the development of a model inactivating system including only ascorbic acid, iron, and oxygen as necessary components. A similar inactivating process was observed in reconstituted systems using cytochromes P_{450}. Initial amino acid analysis suggested the loss of one histidine residue per subunit in the inactivated enzyme. It is of interest to determine the nature of the structural modification in the inactivated enzyme. Such information would clarify the mechanism of inactivation and enable comparison of products of various *in vitro* inactivating systems as well as *in vivo* isolates. Such information will facilitate a determination of the relevance of the *in vitro* inactivating systems to the *in vivo* pathway of protein turnover in general and glutamine synthetase degradation in particular.

Major Findings

1. Attempts to adapt a specific fluorometric histidine assay for use with acid hydrolysates were unsuccessful (J. Farber), but the loss of one histidine per subunit was confirmed by careful amino acid analysis (R. Levine).

2. Previous work in this laboratory had indicated that subtilisin initially cleaves glutamine synthetase in two and that the cleavage products can be separated by exploiting differences in their molecular weights. This procedure was applied in our project with a view toward using the cleavage products of the inactivated glutamine synthetase for structural analysis. Using high performance liquid chromatography gel filtration in SDS, it was found in the case of the native, active enzyme that loss of activity was exactly coincident with loss of intact subunit. The simplest explanation is that the initial single clip results in loss of enzymatic activity.

3. Subtilisin was found to cleave the ascorbate inactivated glutamine synthetase in two pieces of molecular weight similar to those of the subtilisin fragments of the native enzyme. Of interest in light of the possible physiologic role of the inactivated enzyme in the *in vivo* degradative pathway is that the inactivated enzyme is significantly more susceptible to degradation by subtilisin than is the native enzyme.

4. Ammonium sulfate had been previously noted to be effective in inhibiting the proteolytic inactivation of glutamine synthetase by subtilisin, presumably though not necessarily by the binding of the ammonium to the substrate site. Ammonium sulfate was found to dramatically retard proteolysis of the inactivated glutamine synthetase as well, though the inactivated enzyme continued to be proteolyzed more rapidly than the native enzyme under identical conditions. Using a tripeptide substrate CBZ-gly-gly-leu-pNAN, ammonium sulfate was shown to have no effect on the activity of subtilisin, implying that its effect in the protection of the native and inactivated glutamine synthetase is through an interaction with the substrate (GS) and not with the protease.

5. Proteolytic inactivation of glutamine synthetase by subtilisin, as well as loss of intact subunit, follows apparent first order kinetics. Further investigation of the kinetics of proteolytic inactivation of the native enzyme revealed that the apparent first order loss of activity had been observed using concentra-

tions of glutamine synthetase which were saturating for subtilisin. The K_m of subtilisin for the glutamine synthetase subunit was found to be ~ 0.8 μmolar. The mechanism underlying the apparent first order kinetics of previous experiments is not simply loss of available substrate over time.

Significance to Biomedical Research

Protein turnover is a basic biological process. Oxidative inactivation of glutamine synthetase has been implicated as a preliminary step in the turnover of this enzyme in bacteria. Structural analysis of an oxidatively inactivated form of glutamine synthetase will shed light on the mechanism of inactivation and possibly on a key factor in the control of intracellular protein degradation. Information on the control of protein degradation is of general biomedical interest and recently of particular concern regarding the prevention of the unwanted degradation of the products of cloned genetic material. The study of subtilisin mediated proteolysis of native and modified glutamine synthetase will yield information regarding structural aspects of the two proteins and their interactions with ligands, as well as a more detailed analysis of a proteolytic process. Such processes are widely represented in physiological/pathological phenomena such as intracellular and extracellular protein processing, coagulation, complement activation, and tissue injury.

Proposed Course of Research

Research in the immediate future will concentrate primarily on a structural analysis of the oxidatively inactivated glutamine synthetase. Additional aspects of the interaction between subtilisin and the native and inactivated glutamine synthetase will be studied and exploited as appropriate.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00244-01 LB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Oxidative Inactivation of Enzymes

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

P.I.:	Laura Fucci Laccetti	Visiting Fellow (E.O.D. 1/15/81)	LB NHLBI
Other:	Earl R. Stadtman	Chief, Laboratory 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 20205

TOTAL MANYEARS: 0.8	PROFESSIONAL: 0.6	OTHER: 0.2

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Previously, it was demonstrated that purified glutamine synthetase from E. coli undergoes oxidative inactivation by different enzymatic and nonenzymatic systems. This inactivated enzyme is more susceptible to proteolytic digestion by known proteases and protease preparations from E. coli. Studies in this laboratory demonstrated that NADH-diaphorase is the simplest enzymatic system which catalyzes oxidative inactivation of glutamine synthetase. This inactivation is qualitatively similar to inactivation catalyzed by the microsomal cytochrome P_{450} system, Pseudomonas putida mixed function oxidase system and ascorbate system. Studies have been undertaken to test the capacity of NADH-diaphorase to inactivate enzymes other than glutamine synthetase in order to test the generality of this phenomenon. Techniques used in these studies have included polyacrylamide gel electrophoresis, chromatographic techniques, enzymatic assay of functional proteins, and high pressure liquid chromatography.

PHS-6040
(Rev. 2-81)

Project Description

From *in vivo* and *in vitro* experiments (Levine *et al.*, Proc. Natl. Acad. Sci. 78: 2120-2124, 1981 and Oliver, *et al.*, in Ornston, L. N. (ed.): *Experiences in Biochemical Perceptions*, New York, Academic Press, in press), there is evidence that glutamine synthetase modified by oxidative inactivation is more susceptible to proteolytic degradation. Several enzymic systems (rabbit microsomal cytochrome P_{450} mixed function oxidation system and *P. putida* mixed function oxidation system) and several nonenzymic systems (ascorbate, Fe(II), O_2) are able to oxidatively inactivate glutamine synthetase with the qualitatively similar characteristics. Because NADH-diaphorase-O_2 represents the simplest enzyme system which catalyzes this reaction, we are testing the capacity of this enzyme to catalyze a similar inactivation of other enzymes in order to test the generality of this phenomenon.

Major Findings

We found that diaphorases from microorganisms and pig heart inhibit glutamine synthetase from *E. coli* and from rat liver.

E. coli glutamine synthetase inactivation by microbial diaphorase is dependent on NADH or NADPH, Fe(III) and O_2. It is inhibited by $MnCl_2$, EDTA *o*-phenanthroline, and catalase. However, unlike other inactivating systems, inactivation is prevented by superoxide dismutase and horseradish peroxidase has no effect.

These results suggest that oxidative inactivation of glutamine synthetase might occur by a slightly different mechanism in different systems.

Amino acid analysis of diaphorase inactivated glutamine synthetase revealed that a single histidine per subunit was modified, and the result was the same as that observed with ascorbate inactivated glutamine synthetase.

Under conditions of glutamine synthetase inactivation, microbial NADH-diaphorase was unable to oxidatively inactivate β-galactosidase, β-glucuronidase, alkaline phosphatase from *E. coli* and lysoenzyme from egg white.

Proposed Course of Research

The major goal of this study is to determine if enzymes other than glutamine synthetase are oxidatively inactivated. A survey of approximately twenty enzymes, including glucose-6-P dehydrogenase, alcohol dehydrogenase, pyruvate kinase, etc. is planned.

Publications

None

ANNUAL REPORT OF THE CARDIOLOGY BRANCH,
National Heart, Lung, and Blood Institute
October 1, 1980 through September 30, 1981

The areas of experimental interest of the Cardiology Branch relate mainly to the pathophysiology and treatment of coronary artery disease, valvular heart disease, and of hypertrophic cardiomyopathy, and to the development of non-invasive techniques to assess cardiac structure and function. We have continued to pursue the major new direction of research launched last year; namely, defining 1) the role of calcium channel blocking drugs in the treatment of coronary artery disease and of hypertrophic cardiomyopathy, and 2) the mechanisms of action responsible for the beneficial effects of these drugs. We also have concentrated efforts this year on investigating the conditions leading to abnormalities in left ventricular diastolic function, as well as on defining the clinical significance of these abnormalities and the basic mechanisms responsible for them.

CALCIUM CHANNEL BLOCKING DRUGS

Calcium Channel Blocking Drugs in the Treatment of Hypertrophic Cardiomyopathy (HCM)
We previously hypothesized that myocardial intracellular calcium overload may be a possible etiologic mechanism responsible for the hemodynamic abnormalities of pts with HCM. Last year we demonstrated that verapamil decreased LV outflow obstruction, increased exercise capacity, and improved symptomatic status. This year, we completed several studies that have added to our understanding of the mechanisms responsible for these beneficial effects.

LV outflow tract obstruction: To determine the mechanisms responsible for reducing left ventricular outflow tract gradient, pts were studied at cardiac catheterization with a scintillation probe before and after serial infusions of verapamil. Despite decreases in systemic arterial pressure, verapamil increased LV end diastolic volume by 28% ($p<.01$), and end systolic volume by 118% ($p<.01$). It also decreased LV ejection fraction (from 79 to 67%, $p<.01$). These changes were associated with a reduction in LV outflow tract gradient (from 56 to 12 mmHg, $p<.01$) and an increase in LV peak filling rate (from 3.7 to 4.7 EDV/sec, $p<.05$). It is believed that obstruction to LV outflow is caused by Venturi forces impelling the anterior mitral valve leaflet toward the ventricular septum and into the outflow tract during systole. The changes induced by verapamil would attenuate these forces, since their magnitude is related to the velocity of blood coursing through the outflow tract. Thus, the improved LV filling caused by verapamil probably led to the observed increase in LV volume, which presumably increased the narrowed LV outflow tract found in HCM. The larger outflow tract, in association with the drug-induced decrease in contractile state, would diminish the velocity of blood flow through the outflow tract and thereby reduce the Venturi forces. It therefore seems likely that one of the mechanisms whereby verapamil decreases LV outflow tract gradient and improves symptoms is to facilitate LV filling.

LV pressure volume relations: Last year we demonstrated that verapamil improves LV diastolic filling, assessed noninvasively by radionuclide angiography, in many pts with HCM. To determine whether these relative volume changes are associated with improved pressure-volume relations, we generated high temporal resolution pressure volume loops, using micromanometer catheters and an ECG gated scintillation probe, in pts before and after iv verapamil. Our preliminary data indicate that when verapamil improves peak filling rate, LV end diastolic pressure

decreases and the rate of LV relaxation increases. In addition, the pressure volume curve is shifted downward to the right throughout diastole, so that there is a lower LV diastolic pressure for any given LV diastolic volume. In contrast, neither LV end diastolic pressure nor the rate of LV relaxation improves in those pts with no improvement in peak filling rate. Thus, verapamil-induced improvement in LV rapid diastolic filling appears to be caused by an enhancement of LV relaxation. Moreover, these salutary changes occurring in early diastole are associated with changes in end diastole that appear to reflect an enhancement of LV compliance.

Relative effects of verapamil and nifedipine: Several calcium channel blocking agents are available for clinical use. Although each of these agents inhibits voltage dependent calcium channels, they are not identical in the spectrum of their physiologic actions, having different effects and potencies on different tissues. For example, verapamil reduces sinus node automaticity and impairs AV nodal conduction; nefidipine has neither of these actions. These differences have important clinical implications, since there may be certain circumstances in which the depressant effect on AV conduction might be desired (a pt with atrial fibrillation) and other circumstances in which it might be deleterious (a pt with first or second degree heart block). Therefore, to determine whether nifedipine has comparable beneficial effects to verapamil in pts with HCM, we compared the relative effects of these two calcium blocking agents on LV systolic and diastolic function. We previously found that when given acutely verapamil decreased LV outflow tract gradient and diminished pulmonary capillary wedge pressure. However, while nifedipine increased cardiac output, (from $2.9 \pm .9$ to $3.6 \pm .9$; $p < .001$) it had no effects on filling pressure and on LV outflow tract gradient. Moreover, in contrast to prior studies with verapamil, which improved LV filling, no such change occurred following nifedipine. Additional studies comparing chronically administered verapamil and nifedipine demonstrated that while neither drug altered LV systolic function at rest or exercise, verapamil improved LV diastolic filling at rest to a greater extent than nifedipine. Both drugs enhanced LV diastolic filling during exercise. Thus, while each of the drugs has important hemodynamic effects that may contribute to improved clinical status, these preliminary results suggest that verapamil may prove to be more efficacious, in that it more effectively reduces LV outflow tract gradient and improves LV diastolic function.

Long-term effects of verapamil: We previously showed, using high resolution radionuclide LV time-activity curves, that abnormalities in LV diastolic filling are improved in pts with HCM when verapamil is administered acutely or orally over the course of one week. To determine whether these improvements persist during long-term verapamil therapy, we re-studied 17 pts after one year of therapy, before and after withdrawal from verapamil. We found that although the initial improvement in LV diastolic filling induced by verapamil declines over the course of one year, the drug continues to exert persistent reversible beneficial effects in diastolic filling. This is associated with continued improvement in symptomatic status.

Calcium channel blocking agents in the treatment of coronary artery disease (CAD) Last year we demonstrated that verapamil was an effective anti-anginal agent in pts with incapacitating symptoms due to CAD. We also demonstrated that 90% of all pts with CAD manifest abnormalities in LV diastolic filling, and that verapamil improved filling in the majority of pts.

Ischemia induced abnormalities in LV diastolic function : To categorize further both the effects of ischemia on diastolic function and the mechanisms by which verapamil improves symptoms in pts with CAD, a series of studies was initiated in the Experimental Physiology and Pharmacology Laboratory. In several parallel studies employing total or partial coronary occlusion in dogs, we first demonstrated that myocardial ischemia led to impairment not only of LV global systolic function, but also of LV diastolic function. The latter was characterized by a diminution of the peak rate at which ventricular filling occured, and a prolongation of the time required to achieve peak filling rate. To identify the mechanisms responsible for these global abnormalities, we next examined the regional changes induced by ischemia in the magnitude and timing of changes in LV volume.

Awake dogs were studied 18 days after thoracotomy to implant balloon occluders around the LAD coronary artery. Global and regional cardiac function was assessed with gated blood pool scintigraphy. After coronary occlusion, regional decreases in ejection fraction (systolic function) and increases in peak filling rate (diastolic function) developed within the distribution of the occluded coronary artery. Most interestingly, systole was prolonged in the ischemic region, as estimated by the time to achieve minimum volume. The magnitude of this abnormality in timing was quantitated by generating frequency-distribution curves of time to end systole (number of picture elements with a particular time to end systole plotted against time to end systole). In the nonischemic heart, there is a single-peaked narrow frequency-distribution curve. However, a small ischemic zone, produced by occluding the LAD distally, led to a distinct second population of times to end systole; the relative number of picture elements falling within this second peak (percent mismatch compared to control) was 47%. A larger ischemic zone, produced by a more proximal LAD occlusion, led to a 71% mismatch. These results indicate that LV ischemia significantly prolongs regional systole so that emptying of the ischemic region is occurring during the filling phase of the nonischemic portion of the LV. This mechanical alteration undoubtedly contributes to the impairment of global diastolic filling seen following acute coronary occlusion.

The next step was to determine the possible molecular mechanisms responsible for these abnormalities. It has been shown that ischemia increases myocardial calcium uptake, presumably by impairing the capacity of cell membranes to regulate ion flux. It is therefore possible that excessive calcium uptake by myocardial cells is responsible for some of the ischemia-induced abnormalities in diastolic function. To test this hypothesis, we examined the effects of the calcium blocking agent verapamil on these ischemia-induced abnormalities. Twelve dogs were studied a mean of 18 days after implantation of balloon occluders on the circumflex coronary artery. Gated blood pool scintigraphy was performed before coronary occlusion and after ten minutes of low and high doses of verapamil. Coronary occlusion prolonged the time to end systole in the ischemic region. The percent mismatch between time to end systole of normal and ischemic myocardium was calculated. Low dose verapamil reduced the percent mismatch from 34.6% to 15.8 ($p<.05$) and high dose verapamil reduced it further: from 29.3% to 10.0 ($p<.01$). The improved homogenity of contraction was mainly due to a reduction of the prolonged contraction present in the ischemic portion of the myocardium (268 to 257 msec for low dose verapamil; 274 to 240 msec for high dose verapamil). Thus, verapamil reduces the asynchrony of regional emptying produced by acute myocardial ischemia, a change that probably contributes to the improvement in diastolic function observed following administration of this drug.

This series of experiments therefore demonstrates that: 1) ischemia induces a profound abnormality in global diastolic function; 2) the disorder of global function is caused by regional abnormalities in the time course of myocardial contraction; 3) the abnormality in diastolic filling can be inhibited by a calcium channel blocking drug. This latter finding may have clinical relevance. It also suggests that one of the mechanisms responsible for the diastolic abnormalities caused by ischemia may involve an abnormal influx of calcium into the myocardial cell.

Significance of verapamil-induced depression in function of ischemic myocardium: Another series of experiments examined the myocardial effects of calcium channel blocking agents during partial coronary occlusion, a condition that more closely simulates angina pectoris, rather than acute myocardial infarction. It had previously been shown in our laboratory that verapamil preferentially reduces contractile function of mildly ischemic, versus nonischemic myocardium. If the negative inotropic action of verapamil is a primary effect, it could diminish ischemic injury by reducing the work demands of ischemic myocardium during a period of oxygen deprivation. On the other hand, the negative contractile function observed could be secondary to an increase in ischemia, perhaps caused by the decrease in coronary perfusion pressure resulting from verapamil's systemic hypotensive effects. To determine whether the negative inotropic action of verapamil on ischemic tissue reflects an increase or decrease in ischemic injury, a specially designed fiberoptic probe was employed to measure intramyocardial pH, which was used as a marker of ischemic severity. Verapamil improved pH of ischemic myocardium in 16-open chest dogs: for example, at a dose that decreased mean arterial pressure by 14 mmHg, pH increased from 7.09 to 7.18. The improved pH, presumably reflecting a decrease in ischemia, could have been caused by an increase in blood flow to ischemic myocardium -- however, no such increase was found. Alternative mechanisms responsible for the salutary effects of verapamil include a verapamil-induced 1) decrease in metabolic demands (decreased heart rate, mean arterial pressure, and peak ejection rate), or 2) inhibition of calcium entry into ischemic myocardial cells, an action that would reduce the deleterious effects accruing from the increased intracellular levels of calcium caused by ischemia.

Relative myocardial and vascular effects of verapamil, nifedipine and diltiazem:
There are currently three calcium blocking agents being used clinically. Indirect evidence suggests that the relative vasodilator and myocardial depressant potencies of these drugs may differ. We therefore designed a study to compare definitively the relative effects of verapamil (V), diltiazem (D), and nifedipine (N) on hemodynamics and LV function during partial coronary occlusion. Drug effects were compared at doses of drugs causing either equal decreases in arterial pressure, or matched decreases in coronary vascular resistance. At doses that caused a fall in arterial pressure of 10 mmHg:

	HR	G-EF	LVEDP	PFR	I-EF
P	88	.31	13 mmHG	2.8	.15
V	-20%*	-16%*	+64%*	+40%*	-11%*
D	+34%	-6%	-29%	+1%	-6%
N	+4%	+17%*	-9%	+7%	+40%*

EF=ejection fraction; P=placebo; G=global; i=ischemic myocardium; PFR=peak diastolic filling rate, in end diastolic vols/sec; *=p<.05 vs P; %=change from P.

These three calcium blocking agents were thus found to have different potencies on LV systolic and diastolic function, compared to their relative vasodilator potencies. In particular, verapamil produced the greatest negative inotropic effect; however, it also manifested the most pronounced beneficial effect on

LV diastolic filling. The differences observed among these three agents will undoubtedly have important clinical implications.

CORONARY ARTERY DISEASE

Abnormalities in LV diastolic filling We demonstrated last year that abnormal LV filling at rest [reduced LV peak filling rate (PFR) or increased time to PFR] occurred in 90% of all patients with CAD. Studies in the Experimental Physiology and Pharmacology Laboratory (see above) indicated that LV ischemia prolongs regional systole, a change that must contribute to impaired global diastolic filling. To determine whether similar abnormalities account for the impaired diastolic filling in pts with chronic stable CAD, regional function was analyzed in 11 pts who manifest impaired apical contraction at rest. Global PFR was reduced. Regional analysis indicated that times from the R wave to minimal LV counts (an index of end systole) were prolonged in the apex compared to the base in all CAD pts ($p<.005$), but not in a control population. Further analysis disclosed that diastolic filling was occurring at the base at a time when the apex was still emptying. These data indicate that impaired global LV diastolic filling in CAD pts with regional dysynergy is related in part to regional prolongation in LV systoe.

To determine whether subclinical ischemia due to impaired myocardial flow may be responsible for the abnormalities in LV diastolic function, we studied 16 pts with single vessel CAD before and after coronary angioplasty (PTCA). LV diastolic function was abnormal in 12 of 16 pts all of whom had abnormal LV systolic function with exercise. Two days after PTCA, LV diastolic function at rest improved: PFR increased ($2.1\pm.5$ to $2.8\pm.5$ EDV/sec, $p<.001$) and time to PFR decreased (185 ± 23 to 159 ± 18 msec, $p<.001$). These data suggest that abnormalities of resting LV diastolic function are not fixed, but are a manifestation, at least in part, of reversible myocardial ischemia.

Prognostic importance of LV ejection fraction response to exercise To determine whether LV ejection fraction response to exercise, a measure of reversible LV ischemia, provides information of prognostic importance, we studied 138 pts with CAD and mild angina with RN angiography. Rest or exercise LV ejection fraction was no different among the 115 pts who remained stable, 16 pts who required coronary bypass operation (CABG), or 8 pts who suddenly died (SD). However, change in EF from rest to exercise identified pts with different 2-year event rates: only 4 of 58 (7%) pts with positive ΔEF had increased angina pectoris or SD (1 SD), compared to 19 of 80 (24%) pts with negative ΔEF (6 SD, $p<.01$).Life-table analysis indicated that ΔEF was significantly related to subsequent SD and CABG ($p<.05$). Thus the ΔEF from rest to exercise, a reflection of the severity of exercise-induced LV ischemia, helps define subgroups at high and low risk of progressive symptoms and of SD.

Balloon dilatation of the coronary arteries (PTCA) To date 101 pts have undergone PTCA at the NIH. Single vessel CAD was present in 80 pts, two vessel disease in 15 and dilatation of a bypass graft was attempted in 6. Initial angiographic improvement was demonstrated in 74 pts, 70 of whom improved clinically during subsequent follow-up. Fourteen pts developed severe symptoms with documented restenosis within the first three months following angioplasty. Twelve underwent repeat angioplasty, which resulted in improvement in all but two. Fifty-nine consecutive pts were evaluated with RN angiography at rest and during exercise following successful PTCA. EF was unchanged at rest but exercise EF increased from

51% before to 62% after PTCA ($p<.001$). Exercise induced regional dysfunction was present in 94% of pts prior to PTCA and in only 8% following successful PTCA. Restenosis has occurred in 18% of pts; each demonstrated recurrence of ventricular function abnormalities. In pts remaining stable for six months following PTCA, the improved LVEF during exercise has persisted. We conclude that PTCA often leads to initial and persistent (one to two years) angiographic and functional improvement.

Role of Proteolysis in ischemic damage and in healing following acute myocardial infarction (AMI) Biochemical, ultrastructural, and immuno-histological evidence suggest that increased lysosomal hydrolysis may play a causal role in death of myocardial cells that otherwise would have survived an acute but transient ischemic insult. Furthermore, it has been suggested that interventions which stabilize lysosomal enzymes, and thereby interfere with proteolysis, have the potential to salvage ischemic myocardium that otherwise would become necrotic. Last year, however, we demonstrated in both dogs and rats that proteolysis was diminished early after AMI. We also found that leupeptin, an inhibitor of lysosomal and cytoplasmic thiolproteases, although reducing proteolysis following AMI, did not diminish infarct size. This year, further studies were performed to determine both the role of proteolysis on ischemic damage during AMI, and the relation of proteolysis to healing following AMI. To completely suppress proteolysis during AMI, a combination of proteolytic inhibitors (leupeptin, antipain, and pepstatin -- inhibitors of cathepsins, A, B and D and of Ca-activated neutral protease) were administered shortly before and during acute coronary occlusion. Despite almost complete suppression of proteolysis throughout the phase during which cell death develops, no salvage of ischemic myocardium occurred. These findings demonstrate that inhibition of cellular proteolysis is not an infarct sparing mechanism, and that cellular proteases do not appear to contribute to ischemic myocellular death.

To further characterize the changes in proteolysis occurring in the evolution of AMI, we studied the changes in proteolytic activity over the 72 hour period following AMI. We found proteolysis followed a biphasic pattern: it decreases early after AMI, when irreversible damage develops, and later increases markedly, concomitant with infiltration of inflammatory cells. These data suggested that lysosomal proteases, while not contributing to ischemic cell death, may play an important role in the healing phase of AMI; e.g., protelysis of dead myocardium.

Collagen provides a strucural network around heart muscle cells in normal myocardium. Following AMI, proteolysis of dead myocardium occurs, an activity associated with a massive influx of inflammatory cells. To determine whether collagen is also degraded when proteolytic activity increases during the healing phase of AMI, (which might jeopardize infarct wall strength), and if so, whether WBC proteases are implicated, we then studied tissue from the border zone of the infarct, the ischemic zone itself, and the normal zone of rats 24 hours after AMI. Collagen was measured as hydroxyproline. We found that within 24 hours after AMI, there is a significant degradation of large collagen molecules in the infarct region, especially in the border zone where concentration of WBC is highest. Collagen degradation was not seen in rats made leukopenic (WBC less than $300/mm^3$) by prior irradiation, and in rats given ibuprofen, a potent anti-inflammatory agent. These results indicate an important role of WBC proteases in early degradation of collagen after AMI. Additional longer-term studies demonstrated that not only does ibuprofen decrease the degradation of collagen one day after AMI,

but continued administration of the drug significantly enhances collagen deposition, possibly by sparing newly synthesized collagen from continued inflammatory cell proteases. These results suggest a potential role of such anti-inflammatory agents in the healing phase of AMI.

Effects of increased metabolic demands on survival of ischemic myocardium
A rise in heart rate (HR) may increase metabolic rate and therefore flow requirements of ischemic myocardium, thus favoring necrosis after coronary occlusion. This hypothesis assumes that increased HR raises minimal blood flow necessary for tissue survival. To test this assumption we produced coronary occlusion in 12 closed-chest dogs and compared the amount of ischemic damage in control dogs with that of dogs in which HR was increased by pacing for 5 hrs following coronary occlusion. Infarct size in the two groups was measured three days after coronary occlusion. There was no difference in the degree of blood flow reduction produced by coronary occlusion that was associated with subsequent infarction. If increase in metabolic demands produced by increased HR led to additional necrosis, myocardium with relatively high levels of flow would have subsequently infarcted in those animals that were paced. Lack of such change suggests that moderate increase in HR may not raise flow needs in ischemic tissue sufficiently to augment infarct injury. Effects of HR change during coronary occlusion thus may be due chiefly to changes in collateral flow, rather than to deleterious effects of changes in metabolic requirements.

NHLBI Type II Coronary Intervention Study The primary aim of this randomized double-blind prospective study, carried out in collaboration with the Molecular Disease Branch, is to determine whether lowering LDL cholesterol with cholestyramine and diet in pts with premature CAD and Type II hypercholesterolemia will retard the progression of CAD. The major criterion we will employ to answer this question is whether there is regression of anatomic disease or evidence of slower progression, conclusions that will be based on coronary angiograms obtained at initiation into study and after 5 years of treatment. By October, 1981, we will have: 1) concluded data collection for the program; 2) discharged all pts into other programs of NIH or to their source of private care; 3) transferred all basic data to the co-ordinating center. The next year should see closure of the data base and analysis completed. Final closure of the program by July 1, 1982 should be possible.

HYPERTROPHIC CARDIOMYOPATHY

Characterization of hypertrophy in pts with HCM HCM is a disease of cardiac muscle characterised by a hypertrophied nondilated LV. Last year we defined the morphologic distribution of the hypertrophy by two dimensional (2-D) echo in 125 pts. In that study we found four patterns of distribution of LV hypertrophy, ranging from localized to widespread. Pts with widespread hypertrophy had marked functional limitation and more often demonstrated obstruction to LV outflow than subjects with more limited hypertrophy. Because extent and distribution of hypertrophy may relate to propensity to sudden death, these abnormalities were more extensively characterized. Thus, 21 pts without evidence of hypertrophy by M-mode echo were studied by 2-D echo to determine whether they had a form of HCM that could not be detected by conventional M-mode techniques. Each pt was suspected clinically of having HCM because of a distinctly abnormal ECG and either a family history of HCM or cardiac symptoms. Using 2-D echo to reconstruct LV wall geometry, 16 of the 21 pts (76%) had prominent but unusually located regions of LV hypertrophy. In each instance the hypertrophy involved regions of

the LV wall through which the M-mode ultrasound beam does not usually pass; i.e. posterior ventricular septum (7 pts), anterior or lateral LV free wall (7 pts) and ventricular septum near the apex (2 pts). This study indicates that some pts with HCM may have substantial hypertrophy present in unusual locations of the LV wall such that only wide-angle 2-D echo permits definitive confirmation of the diagnosis of HCM. Another unique subgroup of pts with HCM was identified. In each of these pts, hypertrophy was shown by 2-D echo or necropsy examination to be virtually confined to the apical portion of the LV wall, a region inaccessible to conventional M-mode echo. The apical distribution of LV hypertrophy was often associated with: 1) T wave inversion; 2) "hour-glass" angiographic appearance of the LV in systole with mid-cavity obliteration and a small, poorly contractile distal apical segment of LV; 3) mid-ventricular systolic pressure difference between the small distal cavity and larger proximal chamber.

Sudden death in HCM The clinical profile of 78 pts with HCM who died suddenly, was analyzed. At the time of sudden death the majority of pts were young (70% were less than 30 years of age) and without functional limitation (54%). No clinical or morphologic parameter analyzed proved particularly reliable in identifying pts at risk for sudden death, including ventricular septal thickness measurements, ECG, or hemodynamic variables, such as magnitude of LV outflow tract obstruction or LVEDP. However, a prospective study on the significance of arrhythmias detected on 24 ambulatory ECG monitoring yielded important insights as to the mechanisms responsible for, and possible predictors of, sudden death. We previously demonstrated a particularly high prevalence (66%) of high grade ventricular arrhythmias (Grade 3 and above) in 99 pts with HCM, including 19% with episodes of asymptomatic ventricular tachycardia (VT). Because the significance of these arrhythmias in identifying pts at high risk for sudden death was not known, clinical outcome was assessed 3 years after the initial ambulatory ECG. Of the 84 pts who did not undergo operation, 6 died suddenly or experienced cardiac arrest, one died of progressive CHF and the other 77 survived without cardiac catastrophe. While sudden death was relatively uncommon in pts with high grade ventricular arrhythmia other than VT (annual mortality 1%), the finding of VT on 24 hour ECG identified a subgroup of pts at high risk for sudden death (annual mortality 8.6%). Although no conclusions can be drawn regarding the impact of therapy, our finding suggest 24 hour ECG monitoring should be performed in pts with HCM, and that it may be reasonable to initiate antiarrhythmic therapy if VT is identified.

VALVULAR HEART DISEASE

LV dysfunction in pts with mitral regurgitation To determine optimal time for valve replacement in pts with mitral regurgitation (MR), we prospectively studied 21 pts with MR and severe symptoms who subsequently underwent operation. Postop there was a marked reduction in EF at rest (38 ± 10 % postop vs 51 ± 10 preop, $p<.05$) and EF during exercise (41 ± 12 % postop vs 56 ± 12 preop, $p<.01$). EF postop was subnormal (less than 45%) at rest in 15/20 pts and subnormal (less than 55%) during exercise in 16/19. Postoperative EF during exercise was inversely related to preop LV enddiastolic dimension (LVEDD) and end systolic dimension (LVESD), as determined by echo studies at rest. Thus, a higher percentage of pts demonstrated irreversible LV failure (failure of LVEDD to normalize and worsening LVEF at rest and during exercise postop) when preoperative LVEDD was 70 mm or greater, and LVESD was 45 mm or more. 83% of pts with a preoperative LVEDD more than 70 mm have a severely depressed postoperative rest EF compared to only 31% of pts whose preoperative LVEDD was 70 mm or less. The only

operative death in the series occurred in a pt whose preop LVEDD was 94 mm. The long term clinical significance of these postop abnormalities of LV function remains to be determined. In the short term, all pts report symptomatic improvement postop, and there was no correlation between postop EF and exercise capacity (treadmill) postop. Insufficient time has elapsed, however, for conclusions to be drawn about the relation between postop LV function and survival. In summary, our data demonstrate: 1) by the time pts with MR become symptomatic enough to require operation, most have sustained irreversible LV dysfunction which becomes manifest postop; 2) LV dysfunction preop may be masked by the ability of the LV to decompress into a low impedance atrium; 3) preop LV dilatation by echo (LVEDD >70 or LVESD >45 mm) may identify pts at greater risk for severe postop LV dysfunction; 4) the clinical significance of this depressed postop LV function will require long-term follow-up studies.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01661-06 CB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Natural History of Asymptomatic and Mildly Symptomatic Patients with Coronary Artery Disease

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

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

COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

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

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

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

PHS-6040
(Rev. 2-81)

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

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

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

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01670-05 CB

PERIOD COVERED
October 1, 1980 to September 30, 1981

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

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

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

COOPERATING UNITS (if any)

None

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

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

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

PHS-6040
(Rev. 2-81)

Project description: In patients with aortic regurgitation (AR), we presently employ the development of symptoms as the primary indication for proceeding with valve replacement. However, the results of operation are often less than optimal in terms of symptom relief and long-term survival. In order to develop more sensitive indicators of deterioration of cardiac function in asymptomatic patients with AR, extensive clinical and hemodynamic evaluations are being performed. To date, 93 functional class I or II patients with aortic regurgitation have been evaluated with echocardiograms, graded treadmill exercise tests, radionuclide cineangiograms at rest and during supine exercise, measurement of pulmonary artery wedge pressure response to intense supine exercise and 24-hour ambulatory monitoring. The average follow-up time has been 34 months. This group has been divided into 21 asymptomatic patients with mild aortic regurgitation, 59 asymptomatic patients with moderate to severe AR, and 13 patients who have undergone aortic valve replacement after having been initially followed in the Natural History Study. These latter 13 were operated on either because of decreasing left ventricular function or the development of symptoms while under observation. Only one patient from this group has died at this point, and he had mild AR; significant coronary artery disease was found at the time of post-mortem examination.

In comparison to this group, the same intensive evaluation has been carried out on 47 patients who were referred either with moderate to severe aortic regurgitation and significant symptomatology or evidence of left ventricular dysfunction. These patients have had their aortic valves replaced and are being re-evaluated six months after operation. There have been seven deaths in this group. Four were at the time of operation and two occurred within the first month of discharge. Both of these latter patients had also undergone aortic root replacement for aortic root disease.

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

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

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

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

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01672-05 CB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Maintenance of a Computerized Clinical Data Bank for Cardiology Patients

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

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

COOPERATING UNITS (if any)

Surgery Branch, NHLBI

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

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

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

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

PHS-6040
(Rev. 2-81)

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

In the past year, the program has been expanded to include the results of exercise stress testing and electrocardiographic ambulatory monitoring. In the process, forms have been developed for Medical Records so that this information is now recorded in the patients active medical record. This expansion of the data base has obviated the need for special data pools generated by the Biostatistics Department for natural history studies of patients with aortic regurgitation and coronary artery. Furthermore, statistical programs have been incorporated into the data bank to allow for on line statistical analyses and actuarial graphs from the data base.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01719-04 CB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Antiarrhythmic Effects of Verapamil in Patients with Hypertrophic Cardiomyopathy

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

PI:	Douglas R. Rosing	Senior Investigator	CB	NHLBI
Other:	Kenneth M. Kent	Head, Cardiovascular Diagnosis	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.1	.05	.05

CHECK APPROPRIATE BOX(ES)
☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER
☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Verapamil has been successful in treating paroxysmal atrial tachycardia and other forms of supraventricular arrhythmias in patients with hypertrophic cardiomyopathy as well as other forms of heart disease. It has not been of any consistent benefit in patients with significant ventricular arrhythmias.

PHS-6040
(Rev. 2-81)

Project description: Verapamil, a calcium channel blocking agent, is an effective antiarrhythmic agent for supraventricular tachycardias, although less success has been obtained with ventricular arrhythmias. Patients with hypertrophic cardiomyopathy tolerate all forms of tachyarrhythmias very poorly, and ventricular or supraventricular tachyarrhythmias may be a major mechanism for sudden death in this group.

It is well known that verapamil can acutely revert supraventricular tachycardia (SVT) to sinus rhythmn in 80-100% of patients. However, the use of oral verapamil to maintain sinus rhythm in these patients has not been well studied. In seven patients (four without other demonstrable cardiac disease) who were refractory to the usual treatment for SVT, we have been able to eliminate or markedly reduce the frequency of episodes of SVT with the chronic use of verapamil. These patients have been on the drug for periods up to 40 months.

Verapamil administration has been especially helpful in patients with hypertrophic cardiomyopathy who are in atrial fibrillation. We have initiated verapamil treatment in 23 such patients and 20 have had an excellent clinical response for periods up to 30 months. Three of these patients have surprisingly reverted to sinus rhythm and two have remained in this rhythm for periods up to 18 months.

Although two patients with significant (Lown Grade III-IV) ventricular arrhythmias have had a ≥75% reduction in ventricular ectopy with verapamil, many others have had no improvement in their ventricular arrhythmias and have required additional antiarrhythmic therapy.

In summary, verapamil seems to be a very effective antiarrhythmic agent for patients with hypertrophic cardiomyopathy who suffer from atrial tachyarrhythmias. This observation is especially important in those patients with chronic atrial fibrillation who previously required digoxin to slow the ventricular response, since the positive inotropic effect of digitalis glycosides can increase left ventricular outflow obstruction in patients with obstructive hypertrophic cardiomyopathy. The medication does not seem to be as helpful in patients with ventricular arrhythmias, but an evaluation on an individual basis is recommended, for verapamil has been effective in suppressing ventricular arrhythmias in a few patients.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01750-03 CB

PERIOD COVERED
October 1, 1980 to September 30, 1981

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

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

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

COOPERATING UNITS (if any)

None

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: .5	PROFESSIONAL: .4	OTHER: .1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Verapamil has been administered to over 150 patients with hypertrophic cardiomyopathy in order to try to improve their quality of life. Approximately 60% have remained on the medication for at least six months (range 6-45). These patients have manifested an improvement in subjective symptomatic status as well as exercise capacity over this period of time.

PHS-6040
(Rev. 2-81)

Project description: The primary approach to symptomatic therapy in patients with hypertrophic cardiomyopathy (HCM) is the use of beta blocking agents. We previously demonstrated that verapamil reduces left ventricular outflow tract obstruction and improves exercise capacity and subjective symptomatology in hospital in such patients. To assess the chronic effects of verapamil in HCM, over 150 patients whose lifestyle was unacceptable despite propranolol therapy were begun on oral verapamil in hospital between September, 1977 and January, 1981. Less than 10% of patients were not discharged on verapamil due to: side effects (1 death), non-compliance, and symptomatic deterioration. The major reason the drug was stopped in patients discharged on verapamil was because symptoms were unrelieved or recurred. Less than 2% of patients discharged on verapamil had the drug discontinued because of non-physiologic drug side effects. Five patients died while on chronic drug treatment. Approximately 60% of patients discharged on the drug have remained on verapamil (6-45 mos). Adverse hemodynamic effects experienced in the patients included six deaths, six episodes of pulmonary congestion, three of hypotension, two cases of sinus arrest, approximately 10% incidence of development of junctional rhythm or Wenkebach 2 heart block. Non-cardiovascular side effects included upper abdominal discomfort in two patients, frequent constipation, and occasional descriptions of hair loss. In almost all cases of hypotension, junctional rhythm, 2 heart block, and non-cardiovascular problems, the drug was continued at reduced dosage, although therapeutic efficacy was sometimes compromised because of an inability to use higher doses. Of the patients started on verapamil in hospital, approximately 45% have either improved their functional class by at least 1 grade and/or have increased their exercise duration by 15%. Thus, verapamil appears to offer a much needed new therapeutic approach to the treatment of HCM.

Publications: Rosing, D.R., Condit, J.R., Maron, B.J., Kent, K.M., Leon, M.B., Lipson, L.C., and Epstein, S.E. Verapamil Therapy: A New Approach to the Pharmacologic Treatment of Hypertrophic Cardiomyopathy. Circulation: September, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01761-03 CB

PERIOD COVERED October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Percutaneous Transluminal Coronary Angioplasty

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

PI:	Kenneth M. Kent	Head, Cardiovascular Diagnosis	CB	NHLBI
Other:	Douglas R. Rosing	Senior Investigator	CB	NHLBI
	Carolyn J. Ewels	Biologist	CB	NHLBI
	Lewis C. Lipson	Senior Investigator	CB	NHLBI
	Robert O. Bonow	Senior Investigator	CB	NHLBI
	Stephen L. Bacharach	Physicist	NM	CC
	Michael V. Green	Chief, Applied Physics Sec.	NM	CC
	Charles L. McIntosh	Senior Surgeon	SB	NHLBI
	Michael Jones	Senior Surgeon	SB	NHLBI

COOPERATING UNITS (if any)

Department of Nuclear Medicine
Surgery Branch

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: .5	PROFESSIONAL: .3	OTHER: .2

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Non-operative treatment with a balloon catheter to reduce coronary arterial stenoses is being evaluated. Dilatation of the coronary narrowing improves the patients symptoms, objective assessment of exercise capacity and coronary blood flow during exercise.

PHS-6040
(Rev. 2-81)

Project Description: 101 patients have undergone percutaneous transluminal coronary angioplasty (PTCA). All patients had symptoms of angina pectoris prior to the procedure; fourteen patients were mildly symptomatic, 67 had moderate symptoms (angina with usual activities) and 20 patients had severe symptoms including symptoms at rest. Eighty one patients had single vessel coronary artery disease, fifteen had double vessel disease, and in 5 patients PTCA was attempted in a bypass vein graft. Sixty five (65%) patients had initial angiographic improvement in the coronary stenosis. In the remaining 36 patients the arterial stenosis could either not be crossed with the balloon catheter (22 patients), was not compressable (10 patients) when the balloon was inflated to its maximum distending pressure, or inflation of the balloon resulted in arterial dissection and abrupt occlusion of the artery necessitating urgent coronary bypass operation (4 patients). Of the 65 patients in whom there was initial angiographic improvement of the arterial stenosis, 52 were both initially improved and have remained symptomatically stable up to 18 months after the procedure, and 13 developed recurrent severe symptoms with angiographically documented restenosis at the site of the original angioplasty. PTCA was repeated in eleven of the 13 patients. Seven of the eleven patients remain stable after the second PTCA. All cases of restenosis occurred within the first three months after PTCA. Of the stable patients, 27 have returned for coronary arteriograms six months after the initial PTCA, ten have shown a reduction in the degree of stenosis compared to the immediate post-PTCA calculated stenosis (further improvement), 12 remain angiographically stable and five had had $<20\%$ increase in the degree of stenosis.

To determine whether the improvement in the angiographic appearance of the coronary arteries would reduce exercise induced abnormalities in myocardial perfusion and function, perfusion (TL 201) and gated blood pool (Tc99m) scans at rest and exercise were performed prior to, 2 to 21 days after, and six months following PTCA. Before PTCA 55 of the 65 patients who ultimately had a successful angiographic result were stable enough to undergo exercise testing off medication. During exercise 51/55 patients developed angina and 50 of the 55 patients developed left ventricular wall motion abnormalities (ventricular tachycardia terminated exercise in one patient). Left ventricular ejection fraction either remained unchanged or fell in all. PTCA reduced the average stenosis from 84% to 30% and the average gradient across the stenosis was reduced from 63 mm Hg to 20 mm Hg ($p<.01$). Following PTCA, none of these patients developed angina during exercise. Left ventricular function and perfusion were unchanged at rest. During exercise, only three of the 50 patients with prior wall motion abnormalities developed such abnormalities after PTCA. Ejection fraction during exercise increased in each of the patients from the rest value, and was greater than the value during exercise prior to PTCA, in all except three. Average ejection fraction during exercise was $55\% \pm 3\%$ pre-PTCA and increased to $66\% \pm$ post-PTCA ($p<.01$). In all patients who remained symptomatically stable, myocardial function at rest and during exercise was stable at the six months study following PTCA. Thus, the results demonstrated that angiographic improvement obtained with PTCA leads to improved exercise capacity, LV function, and LV perfusion, all presumably reflecting augmentation of functionally important coronary blood flow.

Five major complications of the procedure have occurred. One patient in whom

the procedure was unsuccessful who developed an acute myocardial infarction 12 hours after PTCA. Myocardial infarction was uncomplicated. Three patients had an apparently uneventful angioplasty procedure but post procedure angiography demonstrated arterial (intimal) dissection at the site of the stenosis. Over 15 to 60 minutes, blood flow through the arteries decreased, symptoms and electrocardiographic changes documented myocardial ischemia. In a fourth patient intimal dissection proximal to the stenosis occurred resulting in arterial occlusion. All four patients underwent urgent coronary revascularization operations. No deaths have occurred in the series.

We conclude PTCA can be performed in selected patients with coronary artery disease. Initial angiographic improvement can be obtained in two-thirds of the patients undergoing the procedure. At this point, the complication rate of the procedure appears to be low. Finally, when angiographic improvement occurs, it is accompanied by restoration of functionally important myocardial blood flow during exercise.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01766-03 CB

PERIOD COVERED

October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)

Specificity of Systolic Anterior Motion of the Anterior Mitral Leaflet

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

PI:	Barry J. Maron	Senior Investigator	CB NHLBI
Other:	John S. Gottdiener	Guest Worker	CB NHLBI
	Lowell W. Perry	Staff Cardiologist	Children's Hospital Medical Center

COOPERATING UNITS (if any)

Department of Cardiology, Children's Hospital Medical Center, Washington, D. C.

LAB/BRANCH

Cardiology Branch

SECTION

Clinical Physiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: .11	PROFESSIONAL: .1	OTHER: .01

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Systolic anterior motion of the anterior mitral leaflet (SAM) is not pathognomonic of hypertrophic cardiomyopathy, but is an uncommon finding in a large population of patients with a variety of other cardiac diseases. When SAM occurs it usually coexists with disproportionate septal thickening.

PHS-6040
(Rev. 2-81)

Project description: Systolic anterior motion of the anterior mitral leaflet (SAM) is a characteristic echocardiographic finding in patients with hypertrophic cardiomyopathy and left ventricular outflow obstruction. However, the value of SAM as a diagnostic marker for hypertrophic cardiomyopathy has been questioned, based on reported occurrences of SAM in other heart diseases. To determine the true specificity of SAM (under basal conditions) M-mode echocardiograms from 722 infants, children or adults with a variety of congenital or acquired heart diseases other than hypertrophic cardiomyopathy were reviewed. Ages ranged from 1 day to 75 years (median 40 years). True SAM was present in 22 (3%) of the 722 patients, yielding an overall specificity of 97%. SAM was particularly common in patients with transposition of the great vessels (11 of 52, 21%). When the study group was analyzed excluding patients with transposition of the great vessels, the prevalence of SAM was only 1.6%, giving a speci ficity of 98%. Of note, SAM was rarely found in patients with normal septal-free wall ratios (prevalence of 0.4%; specificity 99%). In conclusion: 1) SAM is an uncommon finding in a large population of patients with a variety of cardiac diseases other than hypertrophic cardiomyopathy; and 2) while SAM is not pathognomonic of hypertrophic cardiomyopathy, it is nevertheless a highly specific marker for that disease.

Publications: Maron, B.J., Gottdiener, J.S., and Perry, L.W.: Specificity of systolic anterior motion of the anterior mitral leaflet (SAM) for hypertrophic cardiomyopathy: Prevalence of SAM in a large population of patients with other cardiac diseases. British Heart Journal 45: 206-213, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01767-03 CB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Distribution of Hypertrophy by 2-Dimensional Echo in Hypertrophic Cardiomyopathy

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

PI:	Barry J. Maron	Senior Investigator	CB	NHLBI
Other:	John S. Gottdiener	Guest Worker	CB	NHLBI
	Stephen 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 20205

TOTAL MANYEARS: .10	PROFESSIONAL: .05	OTHER: .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 patients with hypertrophic cardiomyopathy, hypertrophy is asymmetric but often involves the anterolateral left ventricle and is not limited to the ventricular septum. Functional symptomatic limitation and outflow obstruction is most common in patients with marked, diffuse hypertrophy.

PHS-6040
(Rev. 2-81)

Project description: Wide-angle two-dimensional echocardiography was used to assess the pattern and distribution of left ventricular hypertrophy in 125 patients with hypertrophic cardiomyopathy. Hypertrophy involved substantial portions of both the ventricular septum and anterolateral left ventricular free wall in 54% of the patients, but was confined to the ventricular septum in 39%, and selectively involved only the left ventricular free wall in 7%. The posterior free wall, through which the M-mode beam passes, was usually the least hypertrophied region of the left ventricle.

Four patterns of distribution of left ventricular hypertrophy were identified in the study population. Compared with patients having other patterns of distribution, those patients with the most widespread hypertrophy involving most of the ventricular septum as well as portions of the left ventricular free wall: 1) more commonly experienced moderate to severe functional limitation (40 of 67 or 60% vs 14 of 58 or 24%; $p < 0.001$) and 2) more often demonstrated obstruction to left ventricular outflow under basal conditions (37 of 67 or 55% vs 10 of 58 or 17%; $p < 0.001$). Of note, hypertrophy was limited to the posterior or apical septum or anterolateral free wall in 18% of patients. In these patients conventional M-mode echocardiography failed to identify the presence of hypertrophy, and therefore the diagnosis of hypertrophic cardiomyopathy could be established only by two-dimensional echocardiography.

Hence, in patients with hypertrophic cardiomyopathy, wide-angle two-dimensional echocardiography is capable of detecting myocardial hypertrophy that involves a wide variety of patterns and is more extensive than may be appreciated by M-mode echocardiography. Although left ventricular hypertrophy is "asymmetric" in most patients with hypertrophic cardiomyopathy, it is not usually confined to the septum and often involves the anterolateral left ventricular free wall.

Publications: Maron, B.J., Gottdiener, J.S., and Epstein, S.E.: Patterns and Significance of Distribution of Left ventricular Hypertrophy in Hypertrophic Cardiomyopathy: A Wide-Angle Two-Dimensional Study of 125 Patients. Am J Cardiol. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01768-03 CB

PERIOD COVERED October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Cellular Disorganization in the Left Ventricular Wall of Patients with Hypertrophic Cardiomyopathy

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

PI:	Barry J. Maron	Senior Investigator	CB	NHLBI
Other:	William Roberts	Head, Pathology Branch	PB	NHLBI

COOPERATING UNITS (if any)
Pathology Branch, NHLBI, NIH

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: .10	PROFESSIONAL: .05	OTHER: .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)

Using a quantitative method to assess the arrangement of cells in ventricular myocardium, we found that disorganized cardiac muscle cells are usually diffusely distributed throughout the septum and left ventricular free wall of patients with hypertrophic cardiomyopathy.

PHS-6040
(Rev. 2-81)

Project description: Hypertrophic cardiomyopathy is a disease of cardiac muscle in which premature sudden death often occurs. Marked disorganization of cardiac muscle cells in the ventricular septum is a highly specific and sensitive marker of hypertrophic cardiomyopathy. Extent and distribution of cellular disorganization in the left ventricular wall was assessed quantitatively in sections of ventricular septum and anterior and posterior left ventricular free wall from 52 patients with hypertrophic cardiomyopathy and 83 patients with other cardiac diseases, using substantial photographic enlargement and video planimetry. In patients with hypertrophic cardiomyopathy, ventricular septal disorganization was extensive (mean area disorganized 36 ± 4%). Disorganization was also marked in the left ventricular free wall (anterior and posterior), mean 26 ± 4%, and was as extensive in anterior left ventricular free wall (mean 32 ± 5%) as in ventricular septum. In control patients, mean area of left ventricular free wall disorganized was only 2%. The most marked degree of left ventricular free wall and combined ventricular septal and left ventricular free wall disorganization (mean 43 ± 6%) was present in 15 patients with hypertrophic cardiomyopathy (≤25 years of age) in whom sudden death was the initial manifestation of heart disease. Hence: 1) in most patients with hypertrophic cardiomyopathy cellular disorganization is diffusely distributed in both ventricular septum and left ventricular free wall, and 2) this distribution of disorganization represents a diffuse cardiomyopathic process, particularly in young, previously asymptomatic patients who die suddenly.

Publications: Maron, B.J., Anan, T.J., and Roberts, W.C.: Quantitative analysis of the distribution of cardiac muscle cell disorganization in the left ventricular wall of patients with hypertrophic cardiomyopathy. Circulation 63: 882-894, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01770-03 CB

PERIOD COVERED

October 1, 1980 to September 30, 1981

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

PI:	John Brensike	Coordinator	CB	NHLBI
Other:	M. Myrianthopoulos	Dietician	CB	NHLBI
	Beverly Rogers	Registered Nurse	CB	NHLBI
	Stephen Epstein	Chief, Cardiology Branch	CB	NHLBI
	Robert Levy	Director, NHLBI	CB	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Cardiology Branch

SECTION

NHLBI, Type II Coronary Intervention Study

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2 1/4	1 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)

The primary aim of the study is to determine whether lowering LDL cholesterol in patients with premature coronary artery disease and Type II Hyperlipidemia will slow, stop or reverse the progression of coronary artery disease as demonstrated by angiographic change.

Other areas of interest which the program should contribute to are:

1) Is angiography a reliable and useful tool for the evaluation of coronary artery disease ?

2) Natural history and survival information in a group of high risk well studied patients ?

PHS-6040
(Rev. 2-81)

Project Description: During fiscal year 1981 we have:

1) Concluded data collection for the program.

2) Discharged all participants into other programs of N.I.H. or back to their source of private care.

3) Noted no significant untoward side effects.

4) Data transfer of all basic data to the coordinating center continues to be timely and complete.

5. Discussion of the detailed analysis of program data has begun.

6. All staff except for the Program Director have been transferred to other programs.

The next year should see closure of the data base and analysis completed, papers written and final closure of the program by July 1, 1982 should be possible.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01792-02 CB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Ventricular Septal Contour in Hypertrophic Cardiomyopathy: Two-Dimensional Echo Analysis

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

PI:	Barry J. Maron	Senior Investigator	CB	NHLBI
Other:	Timothy P. Blair	Guest Worker	CB	NHLBI
	John S. Gottdiener	Guest Worker	CB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Clinical Physiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: .7	PROFESSIONAL: .6	OTHER: .1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Two-dimensional echocardiography permits non-invasive assessment of ventricular septal contour. A variety of septal contours are present in patients with hypertrophic cardiomyopathy which are highly specific markers for that disease.

PHS-6040
(Rev. 2-81)

Project description: Hypertrophic cardiomyopathy is a disease of cardiac muscle in which the ventricular septum is usually involved prominently in the cardiomyopathic process. Because ventricular septum contour may have implications regarding diagnosis and advisability of operation, 70 patients with hypertrophic cardiomyopathy and 57 patients with other heart diseases or normal hearts were studied by two-dimensional echo using primarily parasternal long axis and apical views. A variety of contours were identified, with the most common (25 patients, 35%) being "lemon-shaped" (convex to both left ventricle and right ventricle). Ventricular septum contour in other patients were convex toward left ventricle and flat (30%) or concave (25%) to right ventricle, or flat toward left ventricle and convex to right ventricle (10%). In contrast, in each control patient the right and left septal surfaces were parallel. Patients with hypertrophic cardiomyopathy also showed variable distribution of ventricular septum hypertrophy. In most patients (59, 84%) hypertrophy involved all of ventricular septum and was maximal in the middle 1/3. Of note, in 8 (12%) of patients hypertrophy was discretely confined to the cephalad 1/3 of ventricular septum resembling a "tumor nodule", and in 3 (4%) patients was present only in apical regions. Hence: in patients with hypertrophic cardiomyopathy 1) a wide variety of septal contours are present that represent highly specific echo markers for that disease; 2) defining the distribution ventricular septum hypertrophic may be useful in the planning for septal myotomy and myectomy.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01793-02 CB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

24-Hour Ambulatory ECG Monitoring as a Predictor of Sudden Death in Hypertrophic Cardiomyopathy

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

PI:	Barry J. Maron	Senior Investigator	CB	NHLBI
Other:	Daniel D. Savage	Guest Worker	CB	NHLBI
	Stephen 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 20205

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

"High-grade" ventricular arrhythmias are common in patients with hypertrophic cardiomyopathy. The the presence of ventricular tachycardia was predictive of sudden death in a population of patients followed for 3 years after the initial 24 hour ambulatory ECG monitoring.

PHS-6040
(Rev. 2-81)

Project Description: The prevalence and prognostic significance of ventricular arrhythmias identified by 24-hour ambulatory electrocardiographic monitoring was prospectively assessed in 99 patients with hypertrophic cardiomyopathy. In the absence of antiarrhythmic therapy, high-grade ventricular arrhythmias (grade 3 and above) were common--i.e., identified in 66% of the patients, including 19% with episodes of asymptomatic ventricular tachycardia.

Clinical outcome was assessed three years after the initial 24-hour ambulatory electrocardiogram. Of the 84 patients who did not undergo ventricular septal myotomy-myectomy, six had died suddenly or experienced cardiac arrest, one died of progressive congestive heart failure and the other 77 have survived without a cardiac catastrophe. The prevalence of sudden death or cardiac arrest during the follow-up period was the same in patients with high grade arrhythmias other than ventricular tachycardia (1 of 37 or 3%) as in those with no or low-grade arrhythmias (1 of 29, or 3%). However, the occurrence of sudden cardiac catastrophe was significantly more common in those patients with asymptomatic ventricular tachycardia of brief duration on their 24-hour electrocardiogram (4 of 17 or 24%) than in those patients without ventricular tachycardia (2 of 66 or 3%; $p<0.02$).

In summary: 1) high-grade ventricular arrhythmias are commonly found in patients with hypertrophic cardiomyopathy by continuous 24-hour electrocardiographic monitoring; and 2) while sudden death is relatively uncommon in patients with high-grade ventricular arrhythmias other than ventricular tachycardia (annual mortality 1%), the finding of ventricular tachycardia on 24-hour electrocardiogram identifies a subgroup of patients at high-risk for sudden death (annual mortality 8.6%). Although no conclusions can be drawn regarding the impact of therapy, our findings suggest that 24-hour electrocardiographic monitoring should be performed in patients with hypertrophic cardiomyopathy and that it may be reasonable to initiate anti-arrhythmic therapy if ventricular tachycardia is identified.

Publications: Maron, B.J., Savage, D.D., Wolfson, J., and Epstein, S.E.: Prognostic Significance of 24-hour Ambulatory Electrocardiographic Monitoring in Patients with Hypertrophic Cardiomyopathy: A Prospective Study. Am J Cardiol. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01794-02 CB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Clinical Utility of Plasma Verapamil Levels in Patients with Hypertrophic Cardiomyopathy

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

PI:	Douglas R. Rosing	Senior Investigator	CB	NHLBI
Other:	Martin B. Leon	Clinical Associate	CB	NHLBI
	John R. Condit	Biologist	CB	NHLBI
	Taysir M. Jaouni	Chemist	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

Chemistry Branch

LAB/BRANCH
Cardiology Branch

SECTION
Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

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

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)

Plasma verapamil levels were measured using a highly sensitive assay employing high pressure liquid chromatography and fluorometric detection in all patients with hypertrophic cardiomyopathy on chronic oral verapamil therapy. There was marked interpatient variability in verapamil levels at each dosage and no differences between clinical responders, nonresponders, and patients with serious cardiac side effects related to verapamil. Plasma verapamil levels, however, do have limited usefulness for monitoring therapy in patients with hypertrophic cardiomyopathy.

PHS-6040
(Rev. 2-81)

Project Description: A highly sensitive, reproducible high pressure liquid chromotography assay was used to determine the importance of plasma verapamil levels (PVL) in guiding therapy of pts with hypertrophic cardiomyopathy (HCM). In 81 pts taking oral verapamil (V), there was marked variability between pts in PVL for each dosage.

dose (mg/day)	240	320	360	480	640
mean PVL±2SD (ng/ml)	84±72	119±202	163±172	174±208	256±170

In contrast, variability in peak and trough PVL for a given patient was relatively small (V peak/trough ratio for 23 pts=1.7±.1). PVL in 27 clinical responders (R), with improvement in NYHA functional class and >15% increase in exercise capacity were not different from 15 nonresponders (NR) (R=153±11, vs NR=175±18 ng/ml; NS). Moreover, PVL in 24 pts with serious electrophysiologic or hemodynamic side effects from V ranged from 30 to 540 ng/ml and the mean PVL (201±23 ng/ml) was no different from the R or NR. Thus, in pts with HCM on V therapy 1) there is marked interpatient variability in PVL, which may be due to differences in 1st pass hepatic metabolism; 2) PVL are similar in R, NR, and pts with serious side effects. Although these results indicate PVL are therefore of limited usefulness in predicting therapeutic or toxic effects in pts with HCM, the measurement of blood levels has been helpful in guiding therapy. Several patients have demonstrated very low plasma levels on accepted therapeutic doses of verapamil, and the dose of the drug was increased with improvement in symptoms in some patients. Two patients have continued to have negligible blood levels even at 120 mg q.i.d. of the drug, and are presently undergoing work-up for the explanation of such a finding. At the other end of the spectrum, we have not increased verapamil dosage in patients who have levels already greater than 300 ng/ml.

Publications: Jauoni, T.M., Leon, M.B., Rosing, D.R., and Fales, H.M.: Analysis of verapamil in plasma by liquid chromatography. J of Chromatography 182: 473, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01795-02 CB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Clinical Efficacy of Verapamil Alone and Combined with Propranolol in Treating Patients with Chronic Stable Angina Pectoris

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

PI:	Douglas R. Rosing	Senior Investigator	CB	NHLBI
Other:	Martin B. Leon	Clinical Associate	CB	NHLBI
	Robert O. Bonow	Senior Investigator	CB	NHLBI
	Lewis C. Lipson	Senior Investigator	CB	NHLBI
	John R. Condit	Biologist	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Br.	CB	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Cardiology Branch

SECTION
Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.3	.2	.1

CHECK APPROPRIATE BOX(ES)
☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

An in-hospital study involving 12 patients with chronic stable angina pectoris was undertaken to determine the effects of verapamil given alone and combined with propranolol. Patients improved exercise capacity with verapamil compared to placebo or propranolol and increased exercise time even further with the combination of verapamil and propranolol. Nearly three-quarters of patients started on verapamil alone or in combination with propranolol have received symptomatic benefits for up to 18 months.

PHS-6040
(Rev. 2-81)

<u>Project Description</u>: To determine the effectiveness of oral verapamil (V) alone and with propranolol (P) in pts with chronic angina pectoris (AP), upright bicycle exercise testing (ExT) was performed in 12 pts (9 refractory to beta-blockers), 29-62 yrs, in an inpatient, single-blinded crossover study. Compared to placebo (PL), V (480 mg/d) improved exercise time (ET) in all pts (from 307±40 to 508±42 sec; $p<.001$) and was more effective than P. V+P further increased ET (to 590±63 sec; $p<.01$ vs V) and 9/11 pts (82%) were painfree during ExT (in contrast to 2/11 with P and 1/11 with V). Time to 1 mm ST-segment depression was increased by both V (from 178±21 to 358±50 sec; $p<.05$) and V+P (476±93 sec; $p<.05$) but not P. At the workload causing angina when on PL, V decreased heart rate (HR) (from 112±3 to 104±3; $p<.05$) and tended to decrease pressure-rate product (PRP) (from 18,260±780 to 15,930±940; $p<.1$) while V+P further decreased HR (to 76±3; $p<.001$) and PRP (to 9,660±510; $p<.001$). Training effect was absent, as determined by comparable ExT on no medications, before study, during PL and after study. Thus, in the short-term study of pts with stable AP (1) V was a more effective anti-anginal agent than P and (2) V+P was well tolerated and provided additional improvement in exercise capacity over either drug alone. Nine of the original twelve patients have remained on verapamil alone or in combination with propranolol for periods of time between 14 and 18 months because they have had a significant improvement in their quality of life.

In addition, 28 other patients with symptomatic coronary artery disease were started on verapamil alone, or in combination with propranolol, because they considered their lifestyle "unacceptable" on beta-blocking agents. Over 75% of these patients have remained on verapamil for periods of time up to 18 months. Thus, verapamil therapy for angina pectoris appears to provide an effective addition to the present treatment of this desease in selected patients.

Publications: Leon, M.B., Rosing, D.R., Bonow, R.O., Lipson, L.C., and Epstein, S.E.: Clinical efficacy of verapamil alone and combined with propranolol in treating patients with chronic stable angina pectoris. Am J Cardiol, September, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01797-01 CB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Hypertrophic Cardiomyopathy with Unusual Locations of Left Ventricular Hypertrophy Undetectable by M-mode Echocardiography

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

PI:	Barry J. Maron	Senior Investigator	CB	NHLBI
Other:	John S. Gottdiener	Guest Worker	CB	NHLBI
	Robert O. Bonow	Senior Investigator	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: .11	PROFESSIONAL: .1	OTHER: .01

CHECK APPROPRIATE BOX(ES)
☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

In the vast majority of patients with hypertrophic cardiomyopathy the diagnosis can be made by M-mode echocardiography because hypertrophy is present in regions of the left ventricule through which the M-mode beam passes. In some patients with hypertrophic cardiomyopathy the hypertrophy is located in regions that the M-mode beam cannot reach but two-dimensional echocardiography can visualize.

PHS-6040
(Rev. 2-81)

Project Description: Twenty-one patients without evidence of hypertrophy by M-mode echocardiography were studied by wide-angle two-dimensional echocardiography to determine if they had a form of hypertrophic cardiomyopathy that could not be detected by conventional M-mode echocardiography. Each patient was suspected clinically of having hypertrophic cardiomyopathy because of a distinctly abnormal ECG and either a family history of hypertrophic cardiomyopathy or cardiac symptoms. Patients were 5-49 years old (mean 16 years) and 16 of the 21 had no functional limitation. The most common electrocardiographic abnormalities were deep Q waves, T-wave inversion and right ventricular hypertrophy. Using wide-angle two-dimensional echocardiography to reconstruct the geometry of the left ventricular wall, 16 of the 21 patients (76%) were shown to have prominent but unusually located regions of left ventricular wall hypertrophy. In each instance, the hypertrophy involved regions of the left ventricular wall through which the M-mode ultrasound beam does not usually pass, i.e., posterior ventricular septum (seven patients), anterior or lateral left ventricular free wall (seven patients) and ventricular septum near the apex (two patients). There was no echocardiographic or hemodynamic evidence of left ventricular outflow tract obstruction in any patient.

Hence, some patients with hypertrophic cardiomyopathy may have substantial hypertrophy present in unusual locations of the left ventricular wall. Although electrocardiographic abnormalities suggested the presence of myocardial disease, conventional M-mode echocardiography (performed from standard parasternal positions) did not reliably identify such sites of hypertrophy, which were limited to regions of the left ventricle not accessible to the M-mode beam. Only wide-angle two-dimensional echocardiography permits definitive identification of these unusually located regions of cardiac hypertrophy and confirmation of the diagnosis of hypertrophic cardiomyopathy.

Publications: Maron, B.J., Gottdiener, J.S., Bonow, R.O., and Epstein, S.E.: Hypertrophic Cardiomyopathy with Unusual Locations of Left Ventricular Hypertrophy Undetectable by M-mode echocardiography. Identification by Wide-Angle Two-Dimensional Echocardiography. Circulation 63: 409, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01798-01 CB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Clinical and Morphologic Features of Patients with Hypertrophic Cardiomyopathy and Ventricular Septal Hypertrophy Localized to the Apical Regions of the Left Ventricle ("Apical Hypertrophic Cardiomyopathy")

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

PI:	Barry J. Maron	Senior Investigator	CB	NHLBI
Other:	Robert O. Bonow	Senior Investigator	CB	NHLBI
	William C. Roberts	Chief, Pathology Branch	PB	NHLBI
	Stephen E. Epstein	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 20205

TOTAL MANYEARS: .11	PROFESSIONAL: 0.1	OTHER: .01

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Patients with hypertrophic cardiomyopathy may have hypertrophy confined to the apex of the left ventricle. Identification of this form of the disease can not be made by conventional M-mode echocardiography but requires two-dimensional echocardiography.

PHS-6040
(Rev. 2-81)

Project Description: The clinical and morphologic features of a unique subgroup of patients with hypertrophic cardiomyopathy is described. In each of these patients, ventricular septal hypertrophy was shown by two-dimensional echocardiograpy or necropsy examination to be virtually confined to the apical portion of the left ventricular wall. M-mode echo is unreliable in making the diagnosis of hypertrophic cardiomyopathy since the conventional path of the M-mode beam does not traverse the areas of left ventricular hypertrophy in those patients. Such patients do not appear to be a separate disease entity but rather are a part of the morphologic spectrum of hypertrophic cardiomyopathy, as evidenced by genetic transmission of hypertrophic cardiomyopathy with other patterns of left ventricular hypertrophy identified in family members of these patients and marked disorganization of cardiac muscle cells was present in the left ventricular wall of one patient who died suddenly and was studied at necropsy.

The apical distribution of left ventricular hypertrophy was, in the study patients, often associated with: 1) T wave inversion (although not "giant T wave inversion"); 2) "hour-glass" angiographic appearance of left ventricle in systole with mid-cavity obliteration and a small, poorly contractile distal apical segment of left ventricle; 3) mid-ventricular systolic pressure difference between the small distal cavity and larger proximal chamber.

Of note, our patients with apical distribution of hypertrophy differ in several important respects from nonobstructive "apical hypertrophic cardiomyopathy" described in Japanese patients. This observation suggests that genetic and racial differences may account for the different clinical and morphologic features of this form of hypertrophic cardiomyopathy in the United States and Japan.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01799-01 CB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Sudden Death in Hypertrophic Cardiomyopathy: Clinical Profile in 78 Patients

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

PI:	Barry J. Maron	Senior Investigator	CB	NHLBI
Other:	William C. Roberts	Chief, Pathology Branch	PB	NHLBI
	Stephen E. Epstein	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 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.11	.1	.01

CHECK APPROPRIATE BOX(ES)
☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Patients with hypertrophic cardiomyopathy who die suddenly are most commonly young and asymptomatic. The majority were performing sedentary or mild exertion at the time of death. Hemodynamic findings, ventricular septal thickness and ECG alterations do not appear to predict which patients are at highest risk for sudden death.

PHS-6040
(Rev. 2-81)

Project Description: The clinical profile of 78 patients with hypertrophic cardiomyopathy who died suddenly (or experienced cardiac arrest and survived) was analyzed. At the time of sudden death or cardiac arrest the majority of patients were young (70% were <30 years of age), without functional limitation (54%) and performing sedentary or minimal physical activity (61%). Nineteen (24%) of the 78 patients were taking propranolol in apparently adequate dosages, indicating that this drug does not convey absolute protection against the occurrence of sudden death.

No clinical or morphologic parameter analyzed proved particularly reliable in identifying patients at risk for sudden death. While the vast majority of patients who died suddenly had a markedly increased ventricular septal thickness of >20 mm (48 of 64 patients, 75%), mean septal thickness was similar in patients who died suddenly (25.2 $\pm$ 0.9 mm) and in control patients with hypertrophic cardiomyopathy who have survived (23.6 $\pm$ 0.8 mm). An abnormal electrocardiogram was present as commonly in those patients who died suddenly as in those patients who have survived, i.e., 51 of 53, 96%). In addition, no hemodynamic variable, such as the magnitude of left ventricular outflow tract obstruction or left ventricular end-diastolic pressure, proved to be characteristic of the patients with hypertrophic cardiomyopathy who died suddenly.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01800-01 CB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Clinical Features and Natural History of Hypertrophic Cardiomyopathy in Infants

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

PI:	Barry J. Maron	Senior Investigator	CB NHLBI
Other:	Abdul J. Tajik	Staff Cardiologist	Mayo Clinic
	Herbert D. Ruttenberg	Head, Pediatric Cardiology, Univ. of Utah School of Medicine	
	Thomas P. Graham	Head, Ped. Card., Vanderbilt Univ., Sch. of Medicine	
	Gerald F. Atwood	Pediatric Cardiologist, Vanderbilt Un., Sch. of Med.	
	Benjamen E. Victorica	Pediatric Cardiologist, Univ. of Florida, School of Medicine	
	J. T. Lie	Chief, Department of Pathology	Mayo Clinic
	William C. Roberts	Head, Pathology Branch	PB NHLBI

COOPERATING UNITS (if any)
Pathology Br., NHLBI, Departments of Medicine & Pathology, Mayo Clinic
Dept. of Pediatrics, Vanderbilt University, School of Medicine, Dept. of Ped.
University of Florida, Sch. of Med., Dept. of Ped., Univ. of Utah, Sch. of Med.

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

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

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Infants may manifest all the clinical and structural features of hypertrophic cardiomyopathy in older children and adults supporting the hypothesis that this desease is congenital. While some infants remain without symptoms, those that develop marked symptoms early in life do not usually survive infancy.

PHS-6040
(Rev. 2-81)

Project Description: The clinical and morphologic features and natural history of hypertrophic cardiomyopathy in 20 patients recognized as having cardiac disease in the first year of life are described. The majority of infants (14 of 20) were initially suspected as having heart disease solely because a heart murmur was identified. However, the infants showed a variety of clinical findings, including signs of marked congestive heart failure (in the presence of non-dilated ventricular cavities and normal or increased left ventricular contractility) and substantial cardiac enlargement on chest radiograph. Other findings in the infants differed importantly from those usually present in older children and adults with hypertrophic cardiomyopathy--e.g., right ventricular hypertrophy on electrocardiogram and cyanosis. Consequently, in the majority of these infants (i.e., 14) the initial clinical diagnosis was a congenital cardiac malformation other than hypertrophic cardiomyopathy.

In 12 of the 14 infants who underwent left heart catheterization, substantial obstruction to left ventricular outflow (peak systolic pressure gradient $\geq$35 mm Hg) was present. However, unlike older patients with hypertrophic cardiomyopathy, marked obstruction to right ventricular outflow (35 mm Hg to 106 mm Hg) was also common (nine patients); in six patients the magnitude of obstruction to right ventricular outflow was equal to or exceeded obstruction to left ventricular outflow.

Asymmetric hypertrophy of the ventricular septum relative to the left ventricular free wall was present in the 16 patients who had echocardiographic or necropsy examination. Ventricular septal thickening was substantial in patients studied both before and after six months of age (mean 16 mm), indicating that in patients with hypertrophic cardiomyopathy marked left ventricular hypertrophy may be present early in life and is probably congenital.

Clinical course was variable, but the onset of marked congestive heart failure in the first year of life appeared to be an unfavorable prognostic sign: nine of the 11 infants with congestive heart failure ultimately died within the first year of life. Unlike older children and adults with hypertrophic cardiomyopathy, sudden death in infants (occurring in two patients) was less common than death due to progressive congestive heart failure.

Publication: Maron, B.J., Tajik, A.J., Ruttenbery, H.D., Graham, T.P., Atwood, G.F., Victorica, B.E., Lie, J.T., and Roberts, W.C.: Hypertrophic Cardiomyopathy in Infants: Clinical Features and Natural History. Circulation. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 04001-01 CB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Feline Hypertrophic Cardiomyopathy

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

PI:	Si-Kwang Liu	DVM, Animal Medical Center, New York, New York		
Other:	Barry J. Maron	Senior Investigator	CB	NHLBI
	Lawrence Tilley	DVM, Animal Medical Center,		

COOPERATING UNITS (if any)
Animal Medical Center, New York, New York

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.7	.6	.1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Cats with many of the clinical and morphologic features of hypertrophic cardiomyopathy have been identified. About one-third of such cats show asymmetric septal hypertrophy and marked disorganization of cardiac muscle cells in the ventricular septum, similar to patients with hypertrophic cardiomyopathy.

PHS-6040
(Rev. 2-81)

Project Description: Gross anatomic features and the pattern and extent of cardiac muscle cell disorganization were studied in the hearts of 51 cats with spontaneously occurring hypertrophic cardiomyopathy. Each cat had a hypertrophied, but nondilated left ventricle. Ventricular septal disorganization was extensive, involving 5% or more of the relevant areas of the tissue section, in 14 (27%) of the 51 cats. Marked septal disorganization occurred only in those cats with asymmetric septal hypertrophy (ventricular septal to left ventricular free wall thickness ration of $\geq$ 1.1). Disorganization of cardiac muscle cells was uncommon and less extensive in the left ventricular free wall of the cats with hypertrophic cardiomyopathy. Disorganization involved the free wall of only 7 cats, each with asymmetric septal hypertrophy, and occupied >5% of the free wall tissue sections in just 3. Hence, about one fourth of our population of cats had hypertrophic cardiomyopathy resembling the human form of this disease, with asymmetric left ventricular hypertrophy and marked disorganization of cardiac muscle cells in the ventricular septum. The majority of cats (about 75%), however, demonstrated a form of hypertrophic cardiomyopathy characterized by symmetric ventricular hypertrophy and normal arrangement of cardiac muscle cells.

Publication: Liu, S.K., Maron, B.J., and Tilley, L.: Feline Hypertrophic Cardiomyopathy. Gross Anatomic and Histologic Findings. Am J Pathol 102: 388-395, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 04002-01 CB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Comparative effects of verapamil and nifedipine on exercise capacity and symptoms in patients with hypertrophic cardiomyopathy

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

PI:	D. R. Rosing	Senior Investigator	CB	NHLBI
Other:	K. M. Kent	Head, Cardiovascular Diagnosis	CB	NHLBI
	L. C. Lipson	Senior Investigator	CB	NHLBI
	R. O. Bonow	Senior Investigator	CB	NHLBI
	R. O. Cannon	Clinical Associate	CB	NHLBI
	S. E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.3	.2	.1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Verapamil, a calcium channel blocking agent, has been shown to be an effective new therapeutic agent for the treatment of patients with hypertrophic cardiomyopathy. In order to determine whether other drugs of this type will be equally effective, nifedipine was compared with verapamil and placebo administration in a double-blind randomized study. Early results indicate that both verapamil and nifedipine can improve exercise capacity and symptomatic status in patients with hypertrophic cardiomyopathy, but patients must be carefully chosen because serious adverse drug effects can occur.

PHS-6040
(Rev. 2-81)

Project Description: Verapamil is an effective new therapeutic agent for the treatment of patients with hypertrophic cardiomyopathy. Nifedipine, also a calcium channel blocker, produced different effects than verapamil when given to patients with hypertrophic cardiomyopathy in the catheterization lab. In order to assess the clinical efficacy of nifedipine in these patients, and to compare its effects with those of verapamil, treadmill exercise capacity and subjective symptomatic status was evaluated in 13 patients during in-hospital oral verapamil, nifedipine, and placebo administration. Three patients could not complete the study due to hypotension (verapamil), syncope (nifedipine), and pulmonary edema (nifedipine). On placebo, average treadmill exercise capacity was 5.5±3.3 min. Peak treadmill exercise capacity on verapamil was 7.5 ± 2.9 min and on nifedipine was 7.0 ± 2.8. From a symptomatic standpoint, 5 patients described their "best" drug periods(s) occurring on verapamil, 5 on nifedipine, 3 on placebo. Two patients felt "worst" on verapamil, 5 on nifedipine, and 5 on placebo. Additional serious drug side effects included: Verapamil = pulmonary edema (1), heart block (2), nifedipine = hypotension (1). Six patients were discharged on verapamil and 4 on nifedipine. These findings indicate that both verapamil and nifedipine can improve treadmill exercise capacity and subjective symptomatic status in some patients with hypertropic cardiomyopathy, but patients must be carefully selected because serious adverse drug effects can occur.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 04003-01 CB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Regional Reperfusion Blood Flow and Extent of Myocardial Necrosis

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

PI:	N. J. Davenport	Senior Staff Fellow	CB	NHLBI
Other:	Y. M. Ro	Guest Worker	CB	NHLBI
	R. E. Goldstein	Associate Professor of Medicine and Physiology, USUHS, Bethesda, Md.		
	R. Bolli	Visiting Fellow	CB	NHLBI
	S. E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

Department of Medicine, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20014

LAB/BRANCH

Cardiology Branch

SECTION

Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.35	0.25	.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)

Reestablishment of blood flow within 5 hours of coronary artery occlusion has been associated with increased survival of myocardium. However, since myocardial muscle is irreversibly damaged before blood vessel networks are destroyed, normal flow may resume without associated myocardial salvage. In 7 dogs with closed chest coronary artery occlusion, release of the occlusion after 5 hrs produced normal blood flow in areas that three days later were shown to be necrotic. Normal blood flow in reperfused myocardium does not indicate myocardial viability.

PHS-6040
(Rev. 2-81)

Project Description: High reflow after coronary occlusion has been associated with salvage of ischemic myocardium within the risk region (RR); however, reperfused flows may or may not correlate with infarction or survival of tissue. We produced closed chest coronary occlusions in 7 dogs, with release after 5 hrs. Microspheres were given during occlusion to confirm ischemia, and 30 min and 3 days after reperfusion (R). After 3 days excised hearts were stained to identify the RR. Infarct (I) was defined by triphenyltetrazolium. RR was divided into areas of hemorrhage (H), infarct, surviving tissue medially adjacent (MA) and laterally adjacent to infarct, and medially (MR) and laterally remote from infarct. Normal epicardium (NEpi) and endocardium (NEndo) were sampled. Blood flow in ml/ min/g:

Time after R	H	I	MA	MR	NEpi	NEndo
30 min	.90	1.3	2.1	2.0	1.4	1.7
3 days	.38	.82	.82	.85	1.2	1.2

Hemorrhagic and infarcted areas had normal flows 30 min after reperfusion; surviving regions in risk region had flows twice normal. Three days after reperfusion all but hemorrhagic regions of the risk region had normal flows. Thus, after 5 hr of occlusion, re-establishment of normal flow was not associated with myocardial salvage. At 3 days only areas of hemorrhage could be distinguished by abnormally low flows.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 04004-01 CB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Regional Flow in Ischemic Myocardium: Effects of Tachycardia

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

PI:	Y. M. Ro	Guest Worker	CB	NHLBI
Other:	N. J. Davenport	Senior Staff Fellow	CB	NHLBI
	R. E. Goldstein	Assoc. Prof. of Med. and Physiology USUHS, Bethesda, Maryland		
	R. Bolli	Visiting Fellow	CB	NHLBI

COOPERATING UNITS (if any)
Department of Medicine, Uniformed Services University of the Health Sciences, Bethesda, Md. 20014

LAB/BRANCH
Cardiology Branch

SECTION
Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS: 0.5	PROFESSIONAL: 0.5	OTHER: 0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Myocardial necrosis following myocardial ischemia is thought to be aggravated by tachycardia, a circumstance which increases the oxygen requirements of normal heart muscle. However, in dogs with five hours of ischemia, a heart rate increase from 120 to 150 beats per minute did not increase the amount of necrosis, although it decreased ischemic zone blood flow slightly. Moderate tachycardia in myocardial ischemia may not alter myocardial metabolism or coronary blood flow sufficiently to produce deleterious effects.

PHS-6040
(Rev. 2-81)

Project Description: A rise in heart rate (HR ↑) may increase flow requirements of ischemic myocardium, thus favoring necrosis after coronary occlusion This assumes that HR raises minimal blood flow necessary for tissue survival. To test this assumption we produced closed-chest coronary occlusion in 12 dogs: 5 paced (HR 150) beginning 8 min post-coronary occlusion and 7 control (HR 122±12). Pacing and coronary occlusion were terminated 300 min after coronary occlusion and blood flow was reestablished. Three days later hearts were stained to define infarct (I) and region at risk (RR). Surviving portions of RR were divided according to medial (M) or lateral (L) position within RR and adjacent (A) or remote (R) position relative to I. Normal epicardium (N epi) was sampled. Mean blood flow (ml/min/g, paced/ control): (*p<.05 paced vs. control)

Time PostCO	I	MA	LA	N epi
5 min	.07/.09	.15/.38	.38/.52	1.48/1.40
30 min	.13/.16	.17/.32	.51/.58	1.74/1.50
285 min	.05/.18*	.20/.54*	.37/.68*	1.62/1.37

If increased HR induced a rise in minimal flow necessary for tissue survival it would cause blood flow to infarcted and surviving RR of paced dogs to be higher than corresponding blood flow to control dogs. Lack of such change suggests that moderate HR increase may not raise flow needs in ischemic tissue sufficiently to increase infarct size. Effects of HR↑ during coronary occlusion may be due chiefly to decreased collateral blood flow.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 04005-01 CB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Effects of Verapamil and Nifedipine on Left Ventricular Function in HCM

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

PI:	R. O. Bonow	Senior Investigator	CB	NHLBI
	D. R. Rosing	Senior Investigator	CB	NHLBI
	S. T. Palmeri	Expert	CB	NHLBI
	L. C. Lipson	Senior Investigator	CB	NHLBI
	K. M. Kent	Head, Cardiovascular Diagnosis	CB	NHLBI
	S. L. Bacharach	Physicist	NM	CC
	M. V. Green	Chief, Applied Physics Section	NM	CC
	S. E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

Department of Nuclear Medicine, Clinical Center

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.3	.2	.1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

To compare the effects of verapamil and nifedipine on left ventricular (LV) systolic function and diastolic filling in pts with hypertrophic cardiomyopathy, we assessed radionuclide angiograms in 11 pts during control and during oral therapy with each drug. Neither drug altered LV ejection fraction, at rest or during exercise. Verapamil, but not nifedipine, improved diastolic filling (increased peak LV filling rate and decreased time to peak filling rate) at rest. Both drugs improved diastolic filling during exercise, and the effects of verapamil and nifedipine were not different. These mechanisms may contribute to the clinical improvement of many pts during oral therapy with calcium channel blocking agents.

PHS-6040
(Rev. 2-81)

Project Description: To compare the relative effects of verapamil (V) and nifedipine (N) on left ventricular (LV) systolic function and diastolic filling in pts with hypertrophic cardiomyopathy (HCM), we analyzed radionuclide ventriculograms in 11 pts during placebo (C), oral verapamil (320-480 mg/day), and oral nifedipine (80-120 mg/day). Neither verapamil nor nifedipine altered LV ejection fraction or peak ejection rate at rest or during exercise. However, verapamil improved LV diastolic filling at rest: peak LV filling rate (PFR), measured in end-diastolic volumes (EDV)/ sec, increased (C=3.2±1.3[±1 SD]; V=3.9± 1.2 EDV/sec, $p<0.02$) and time to PFR (TPFR) decreased (C= 196±32; V=174±32 msec, $p<0.05$). Nifedipine did not alter PFR (3.2 ±1.1 EDV/sec) or TPFR (192±38 msec). During exercise to equivalent work loads, both drugs increased PFR [C=6.0±2.3; V=6.6±2.6 ($p<0.05$); N=6.9±2.8 EDV/sec ($p<0.01$)] and decreased TPFR [C=132±32; V=108±25 ($p<0.05$); N=119±13 msec ($p<0.05$)], and the effects of verapamil and nifedipine were not significantly different. Thus, neither verapamil nor nifedipine alter LV systolic function at rest or exercise in pts with HCM. Verapamil improves LV diastolic filling at rest to a greater extent than nifedipine, but both drugs enhance LV diastolic filling during exercise. These mechanisms may account for the clinical improvement of many pts with HCM during oral therapy with verapamil or nifedipine.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 04006-01 CB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Left Ventricular Function During Exercise in Coronary Artery Disease Subgroups

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

PI:	R. O. Bonow	Senior Investigator	CB	NHLBI
Other:	S. L. Bacharach	Physicist	NM	CC
	M. V. Green	Chief, Applied Physics Section	NM	CC
	K. M. Kent	Head, Cardiovascular Diagnosis	CB	NHLBI
	D. R. Rosing	Senior Investigator	CB	NHLBI
	L. C. Lipson	Senior Investigator	CB	NHLBI
	S. E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

Department of Nuclear Medicine, Clinical Center

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

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

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

One hundred pts with coronary artery disease (CAD) without diagnostic Q waves and with normal resting left ventricular ejection fraction (LVEF) were assessed by radionuclide angiography to determine regional and global LV function during exercise. LVEF with exercise was not different from rest in pts with one vessel disease (VD), but was significantly lower in pts with 2VD, 3VD, and left main disease. The prevalence of abnormal regional and global LV exercise responses increased in stepwise fashion from pts with one VD, 2VD, 3VD, through left main disease. Thus, the sensitivity of detecting CAD by radionuclide imaging is related to the anatomic extent of CAD and will therefore be determined by selection of the patient population.

PHS-6040
(Rev. 2-81)

Project Description: To assess regional and global left ventricular (LV) function during exercise in subgroups of pts with coronary artery disease (CAD) and normal LV function at rest, we studied by radionuclide angiography 100 consecutive pts with normal EKG (without diagnostic Q waves) and normal rest LV ejection fraction (EF). Exercise EF was not different from rest EF (53% rest, 52% exercise, N.S.) in pts with one vessel disease (VD), but was significantly lower in pts with 2 VD (55 vs 51%, $p<.01$), 3 VD (55 vs 48%, $p<.001$), and left main disease (58 vs 46%, $p<.01$). The prevalence of abnormal LVEF exercise responses (<5% increase) and regional wall motion abnormalities (WMA) with exercise was related to the extent of CAD:

	#	LVEF	WMA
1 VD	33	75%	84%
2 VD	29	75%	85%
3 VD	27	86%	95%
Left Main	11	100%	100%
Total	100	86%	88%

Thus, in pts with normal rest EKG and rest LVEF, the prevalence of abnormal LVEF and WMA during exercise is related to the anatomic extent of CAD. These data indicate that the sensitivity of detecting CAD by radionuclide imaging in pts without previous infarction is dependent, in large part, upon selection of the patient population.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 04007-01 CB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Improved Left Ventricular Diastolic Filling Following Coronary Angioplasty

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

PI:	R. O. Bonow	Senior Investigator	CB	NHLBI
Other:	K. M. Kent	Head, Cardiovascular Diagnosis	CB	NHLBI
	D. R. Rosing	Senior Investigator	CB	NHLBI
	L. C. Lipson	Senior Investigator	CB	NHLBI
	S. L. Bacharach	Physicist	NM	CC
	M. V. Green	Chief, Applied Physics Section	NM	CC
	S. E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

Department of Nuclear Medicine, Clinical Center

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.1	.05	.05

CHECK APPROPRIATE BOX(ES)

[X] (a) HUMAN SUBJECTS [ ] (b) HUMAN TISSUES [ ] (c) NEITHER

[ ] (a1) MINORS [ ] (a2) INTERVIEWS

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

Left ventricular (LV) diastolic filling is abnormal in many pts with coronary artery disease (CAD) at rest, even in pts with normal resting LV systolic function. To assess the effect of improved myocardial perfusion on these abnormalities, we studied 16 pts by radionuclide angiography before and after coronary angioplasty (PTCA). Two days after PTCA, resting LV ejection fraction (EF) and heart rate were unchanged, but exercise LVEF increased, reflecting reduction in myocardial ischemia compared to pre-PTCA. In addition, LV diastolic filling at rest improved post PTCA (increased peak LV filling rate and reduced time to peak filling rate). Thus, in many pts with CAD and normal resting LV systolic function, abnormalities of resting LV diastolic filling are a manifestation in part of reversible myocardial ischemia.

PHS-6040
(Rev. 2-81)

Project Description: Left ventricular (LV) diastolic filling assessed by high temporal resolution radionuclide angiography, is abnormal at rest in many pts with coronary artery disease (CAD), even in the presence of normal resting LV systolic function. To determine the effects of improved myocardial perfusion on impaired LV diastolic filling, we studied 16 pts with single vessel CAD before and after percutaneous transluminal coronary angioplasty (PTCA). Despite normal regional and global LV systolic function at rest in all pts, LV diastolic filling was abnormal [peak filling rate (PFR)<2.5 end-diastolic volumes (EDV)/sec or time to PFR$>$ 180 msec] in 12 of 16 pts. All pts had abnormal LV systolic function with exercise. Two days after PTCA, LV ejection fraction and heart rate at rest were unchanged from pre-PTCA values, but LV ejection fraction during exercise increased (54 ± 5 before, $62 \pm 5\%$ after, $p<.001$) and LV diastolic filling at rest improved: PFR increased ($2.1 \pm .5$ to $2.8 \pm .5$ EDV/sec, $p<.001$) and time to PFR decreased (185 ± 23 to 159 ± 18 msec, $p<.001$). Thus, reduction in exercise-induced LV systolic dysfunction after PTCA was associated with improved LV diastolic filling at rest. These data suggest that, in many pts with CAD and normal resting LV systolic function, abnormalities of resting LV diastolic filling are not fixed, but are a manifestation, at least in part, of reversible myocardial ischemia.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 04008-01 CB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Body Surface Potential Mapping in Patients with Hypertrophic Cardiomyopathy

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

PI:	R. M. Watson	Clinical Associate	CB NHLBI
Other:	R. O. Bonow	Senior Investigator	CB NHLBI
	B. J. Maron	Senior Investigator	CB NHLBI
	C. J. Ewels	Biologist	CB NHLBI
	L. S. Green	Asst. Prof. Medicine	Univ. of Utah
	R. L. Lux	Assoc. Research Prof. Med., Nora Eccles Harrison Cardiovascular Research and Training Inst. Univ. of Utah	
	J. A. Abildskov	Director, Nora Eccles Harrision Cardiovascular Research and Training Inst., Univ. of Utah	
	S. E. Epstein	Chief, Cardiology Branch	CB NHLBI

COOPERATING UNITS (if any)
Nora Eccles Harrison Cardiovascular Research and Training Institute, University of Utah, Salt Lake City, Utah

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.3	.2	.1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Patients with hypertrophic cardiomyopathy have abnormal electrocardiograms and a high incidence of arrhythmias. An increased incidence of sudden death has also been demonstrated which is likely to be on an arrhythmogenic basis. Body surface potential maps (BSPM) provide a non-invasive assessment of cardiac electrical depolarization and recovery properties and may aid in identifying cardiac states in which ventricular arrhythmias are more likely to lead to sudden death.

Body surface potential maps are generated from 192 ECG leads. A modified 32 lead system was developed as a more clinically useful system which includes all crucial information and eliminates redundancy of information. The BSPM information is stored on a floppy disc and processed at the Cardiovascular Research and Training Institute of the University of Utah. Resultant BSPM's are correlated with clinical information obtained during the in-patient work-up including two dimensional echocardiography, radionuclide cineangiography, cardiac catheterization and 24-hour ambulatory ECG monitoring in hopes of identifying patients at high risk for sudden death to allow for therapeutic intervention.

PHS-6040
(Rev. 2-81)

Project Description: Patients with hypertrophic cardiomyopathy have abnormal electrocardiograms and a high incidence of arrhythmias. An increased incidence of sudden death has also been demonstrated which is likely to be on an arrhythmogenic basis. Body surface potential maps (BSPM) provide a non-invasive assessment of cardiac electrical depolarization and recovery properties and may aid in identifying cardiac states in which ventricular arrhythmias are more likely to lead to sudden death.

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Clinical data of interest include:

(1) the presence or absence of obstruction.
(2) the presence or absence of Q waves on the standard 12 lead ECG.
(3) the pattern of hypertrophy on two-dimensional echocardiography and how it might correlate with activation sequence or recovery properties.
(4) hemodynamic data obtained from cardiac catheterization.
(5) ejection fraction obtained by radionuclide cineangiography.
(6) diastolic filling properties as determined by gated blood pool scanning.
(7) information regarding arrhythmias as detected by 24-hour ambulatory ECG monitoring.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 04009-01 CB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Prognostic Importance of Left Ventricular Ejection Fraction Response to Exercise

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

PI:	R. O. Bonow	Senior Investigator	CB	NHLBI
Other:	K. M. Kent	Head, Cardiovascular Diagnosis	CB	NHLBI
	D. R. Rosing	Senior Investigator	CB	NHLBI
	L. C. Lipson	Senior Investigator	CB	NHLBI
	C. J. Ewels	Biologist	CB	NHLBI
	S. L. Bacharach	Physicist	NM	CC
	M. V. Green	Chief, Applied Physics Section	NM	CC
	S. E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

Nuclear Medicine, Clinical Center

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

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)

To assess the prognostic implications of the left ventricular (LV) ejection fraction (EF) response to exercise, a measure of reversible myocardial ischemia in pts with coronary artery disease (CAD), we studied 138 CAD pts who had mild angina with radionuclide angiography. Rest or exercise LVEF did not identify the 8 pts who subsequently died or the 16 pts who required operation for increased angina during the mean 2 year follow-up period. However, the change (Δ) in EF from rest to exercise was predictive: 7% of pts with positive ΔEF died or underwent operation compared to 24% of pts with negative ΔEF ($p<0.05$). Thus, the absolute level of LVEF at rest or exercise does not predict which pts would progress, but the ΔEF helps to define subgroups at low and at high risk.

PHS-6040
(Rev. 2-81)

Project Description: To determine whether the left ventricular ejection fraction (LVEF) response to exercise, a measure of reversible myocardial ischemia, provides information of prognostic importance, we studied 138 pts with coronary artery disease (CAD) and mild angina with radionuclide angiography. During the mean 2 year follow-up period, increased angina despite medications was the only indication for operation (CABG). Rest or exercise LVEF was not different among the 115 pts who have remained stable, 16 pts who required CABG, or 8 pts who suddenly died (SD). However, change (Δ) in EF from rest to exercise identified pts with different 2 year event rates: only 4 of 58 (7%) pts with positive ΔEF had increased angina or SD (one SD), compared to 19 of 80 (24%) pts with negative ΔEF (6 SD), $p<0.01$. Life-table analysis indicated that ΔEF was significantly related to subsequent SD and CABG ($p<0.05$). Thus, the absolute level of LVEF at rest or during exercise does not predict which CAD pts with mild angina will progress or have SD, but the ΔEF from rest to exercise, a reflection of the severity of exercise-induced myocardial ischemia, helps to define subgroups at high and at low risk.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 04010-01 CB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Clinical Efficacy of Nifedipine With and Without Propranolol in Patients With Exertional Angina Pectoris Due to Coronary Artery Disease

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

PI:	D. R. Rosing and	Senior Investigator	CB	NHLBI
	L. C. Lipson	Senior Investigator	CB	NHLBI
Other:	R. O. Cannon	Clinical Associate	CB	NHLBI
	K. M. Kent	Head, Cardiovascular Diag.	CB	NHLBI
	R. O. Bonow	Senior Investigator	CB	NHLBI
	S. E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

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

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

An in-hospital study has been initiated evaluating the effectiveness of nifedipine therapy alone or in combination with propranolol in patients with chronic stable angina pectoris. Six patients have completed the protocol. Four had their best response from the combination of nifedipine and propranolol.

PHS-6040
(Rev. 2-81)

Project Description: We have previously described that oral verapamil, a calcium channel blocking agent, appears to be as effective as propranolol in improving exercise capacity in patients with chronic stable angina pectoris, and that the combination of verapamil and propranolol produces greater benefits than achieved with the administration of either drug alone. In order to determine whether nifedipine, another calcium channel blocking agent, is as effective an antianginal agent as verapamil, a similar study was initiated comparing the effects of nifedipine, placebo, propranolol, and the combination of nifedipine and propranolol administration to this group. The effects of these drug interventions on exercise capacity, left ventricular systolic function, and left ventricular diastolic function is being examined. So far, six patients have completed the protocol. Four of the six had their best exercise performance while on the combination of nifedipine and propranolol, one on propranolol alone, and one on placebo. Three had their shortest exercise duration on propranolol, two on nifedipine, and only one while on placebo. As more patients complete the study, further information regarding the mechanism of action of nifedipine and its effect on systolic and diastolic function, as well as its relative effectiveness as an antianginal agent, will be forthcoming. Furthermore, as patients are sent home on chronic nifedipine therapy, the long-term effectiveness of this drug as an antianginal agent should also become apparent.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 04011-01 CB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Comparative Effects of Verapamil, Diltiazem and Nifedipine on Hemodynamics and Left Ventricular Function

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

PI:	Joann Urquhart	Staff Fellow	CB	NHLBI
Other:	Randolph Patterson	Senior Investigator	CB	NHLBI
	Stephen Bacharach	Physicist	NM	CC
	Michael Green	Chief, Applied Physics Section	NM	CC
	Edith Spier	Chemist	CB	NHLBI
	Roger Aamodt	Chief, Whole Body Counter Sec.	NM	CC
	Stephen 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 20205

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

There are currently three calcium antagonist drugs--verapamil, diltiazem, and nifedipine--being investigated for clinical use in the treatment of angina. Although each shares a common mechanism (inhibits calcium entry into cells) they appear to have different physiologic effects and potencies on the heart and blood vessels. This study definitely examined this question and demonstrated that at doses causing equal decreases in blood pressure verapamil, diltiazem, and nifedipine had markedly different effects on muscle contraction: nifedipine increased contraction, verapamil decreased contraction and diltiazem had no change. These differences will have important clinical implications in choosing one of these drugs for a particular patient.

PHS-6040
(Rev. 2-81)

Project Description: Although verapamil (V), nifedipine (N) and diltiazem (D) are potent Ca antagonists, with negative inotropic potential, their relative effects on myocardial function in relation to their vasodilator potencies are unknown. To simulate anginal conditions, 19 conscious dogs were studied after partial occlusion (ischemia) of the circumflex coronary artery during placebo (P) (n=5), V(n=5), N(n=4) or D(n=5) therapy. Myocardial flow was measured by microspheres, LV function by radionuclide blood pool scans. Drug effects were compared at doses causing equal decreases in arterial pressure, or example: -10 mmHg:

	HR	G-EF	LVEDP	PFR	I-EF
P	88	.31	13 mmHg	2.8	.15
V	-20% *	-16%*	+64%*	+40%*	-11%*
D	+34% *	-6%	-29%*	+1%	-6%
N	+4%	+17%*	-9%	+7%	+40%*

EF=Ejection Fraction; G=global; I=ischemic myocardium; PFR=peak diastolic filling rate, in end diastolic vols/sec; *=p<.05 vs P; %=change from P. Similar findings were observed at matched decreases in coronary vascular resistance. We conclude the relative potencies of these 3 Ca antagonists drugs on LV systolic and diastolic function during myocardial ischemia are different when compared to their relative vasodilator potencies. These differences have important clinical implications.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 04012-01 CB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Improvement of Ischemia-Induced Delay in Regional Left Ventricular Emptying by Verapamil

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

PI:	Beverly Jones-Collins	Guest Worker	CB	NHLBI
Other:	Michael Green	Chief, Applied Physics Section	NM	CC
	Randolph Patterson	Senior Investigator	CB	NHLBI
	Stephen Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)
Department of Nuclear Medicine, Clinical Center

LAB/BRANCH
Cardiology Branch

SECTION
Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.25	.20	.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 timing of contraction of the main pumping chamber of the heart is delayed in the affected region after a heart attack. We investigated the effect of verapamil, a new calcium antagonist drug, in this setting. Verapamil reduced the delay in timing of contraction in the abnormal region after heart attacks in dogs, thus improving heart function.

PHS-6040
(Rev. 2-81)

Project Description: Impaired left ventricular filling occurs after acute coronary occlusion, a change that may be of clinical importance. Evidence suggests impaired filling is partly due to delayed emptying of the ischemic region. To determine the effect of verapamil, 12 dogs were studied a mean of 18 days after implantation of balloon occluders on the circumflex coronary artery. Dogs were randomized to a control (n=6) or verapamil (n=6) group. Verapamil 30 g/kg was given after coronary occlusion followed by 5 μg/kg/min [mean blood level=44 ng/ml] and 10 μg/kg/min [mean blood level=76 ng/ml]. Gated blood pool scintigraphy was performed before coronary occlusion and after 10 min of low and high dose verapamil. Normal and abnormal regions of interest were defined by regional differences in phase and amplitude maps. Coronary occlusion prolonged time to minimum volume (TMV) in the abnormal region of interest, and a technique to quantitate TMV mismatch between normal and abnormal region of interest was developed. % mismatch was less during low and high verapamil than the corresponding control study (15.8 vs 34.6%, $p<.05$ for low verapamil; 10 vs 29.3%, $p<.01$ for high verapamil. This was due mainly to reduction of TMV in the abnormal region of interest (268 vs 257 msec for low verapamil; 274 vs 240 msec for high verapamil). Thus, verapamil reduces the asynchrony of regional emptying produced by coronary occlusion and thereby may improve diastolic function.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 04013-01 CB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Functional Mapping of Regional Time to Minimum Volume Before and After Coronary Occlusion in the Dog

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

PI:	Michael Green	Chief, Applied Physics Section	NM	CC
Other:	Beverly Jones-Collins	Guest Worker	CB	NHLBI
	Stephen Bacharach	Physicist	NM	CC
	Stephen Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

Department of Nuclear Medicine, Clinical Center

LAB/BRANCH

Cardiology Branch

SECTION

Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.20	.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)

Timing of contraction of various regions of the heart was evaluated in dogs before and after a small and large heart attack. Timing of contraction was delayed after a small and further delayed after a large heart attack. This is a new way to analyze this abnormality in precise quantitative terms.

PHS-6040
(Rev. 2-81)

Project Description: Ischemia-induced changes in global left ventricular (LV) volume indices (e.g., ejection fraction) have been extensively studied. However, little is known about regional changes in timing of events induced by ischemia: e.g., ischemia may prolong regional systole, impinging on and impairing global diastolic filling. Balloon occluders are implanted in 8 dogs proximally and distally on the left anterior descending coronary artery. Gated blood pool scintigraphy was performed 18 days later in the control state, and after low and high occlusion. Low and high occlusion ischemic beds were estimated by post-mortem dye staining (22 and 45% LV). Functional maps of time to minimum volume (TMV) were gènerated from gated blood pool scintigraphy. We found: 1) in the contrtol state TMV is randomly distributed with small variation, 2) low occlusion produces a small focal area of prolonged TMV, 3) high occlusion produces a larger area of TMV prolongation. A distribution function of TMV (number of picture elements with a particular TMV plotted against TMV in msec) in control, low and high occlusions was created for each dog and the % mismatch of low occlusion vs control area and high occlusion vs control area was calculated to quantitate the changes (47 and 71% mismatch). We conclude that LV ischemia significantly prolongs TMV, an abnormality that can be readily quantitated by TMV functional mapping.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 04014-01 CB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Intramyocardial pH, Drug, Radionuclide and Hemodynamic Studies in Myocardial Ischemia

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

PI:	R. M. Watson	Clinical Associate	CB	NHLBI
Other:	R. E. Patterson	Senior Investigator	CB	NHLBI
	D. R. Markle	Investigator	R	BEI
	S. R. Goldstein	Chief, Mechanical Engineering Sec.	R	BEI
	D. A. McGuire	Investigator	R	BEI
	R. Aamodt	Chief, Whole Body Counter Section	NM	CC
	S. E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

Division of Research Services, Biomedical Engineering and Instrumentation, Nuclear Medicine, Clinical Center

LAB/BRANCH

Cardiology Branch

SECTION

Experimental Physiology and Clinical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

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

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A specially designed fiberoptic probe that is capable of monitoring intramyocardial pH is of unique importance to the study of myocardial ischemia. A partial coronary artery occlusion was produced in open chest anesthetized dogs and myocardial blood flow was determined by the use of tracer microspheres. Heart rate, mean aortic pressure, coronary blood flow, coronary perfusion pressure, cardiac output, and peak ejection rate were monitored. Gated blood pool scanning was performed with a gamma camera to examine global ejection fraction, regional wall motion, and regional ejection fraction. The calcium antagonist drug, verapamil, was administered in progressively increasing doses to determine its effects on ischemia. Blood levels of verapamil were drawn and quantitated. Verapamil was found to increase the depressed intramyocardial pH in the ischemic area presumably reflecting a beneficial effect of the drug on the severity of the ischemic insult.

PHS-6040
(Rev. 2-81)

Project Description: Verapamil reduces contractile function of mildly ischemic myocardium. This could be beneficial (negative inotropic effect leading to a decrease in myocardial metabolic requirements, thereby decreasing ischemia) or could be due to increased ischemia (secondary to decreased coronary perfusion). To determine if verapamil improves or worsens ischemia, intramyocardial pH was measured by a specially designed fiberoptic probe in 16 open chest dogs: coronary blood flow (CBF) was decreased to 25% of control. Verapamil or placebo were given in 3 incremental doses. We also measured myocardial flow (MBF) (microspheres) and LV function (gated blood pool scans). Verapamil improved pH of ischemic myocardium at all doses (*=p<.05 by analysis of variance: eg, at dose 2:

	pH	HR	MAP	EF	PER	CBF	MBF
Control Post Occlusion	7.09	127	80	.38	4.4	9	.30
Verapamil	7.18*	110	66*	.37	4.0*	7	.38
Control Post Occlusion	7.15	151	92	.31	3.5	7	-
Placebo	7.03*	143	106*	.23	4.3*	7	-

(HR=heart rate, MAP=mean arterial pressure, EF= ejection fraction, PER=peak ejection rate, MBF= ml/min/g, CBF=ml/min, PER=L/min. Thus, verapamil improves ischemia-induced pH changes. Since no change occurred in ischemic MBF, the improved pH may reflect a decrease in ischemia [caused by decreased metabolic demands (↓HR, ↓MAP, ↓PER], or by inhibition of Ca++ entry into ischemic cells.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 04015-01 CB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Experiments on pathogenesis of cardiac rupture and aneurysm formation following acute myocardial infarction

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

PI:	R. O. Cannon, III	Clinical Associate	CB	NHLBI
Other:	E. Speir	Chemist	CB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Cardiology Branch

SECTION
Animal Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: .8	PROFESSIONAL: .6	OTHER: .2

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Cardiac rupture and aneurysm formation following acute myocardial infarction may be related to degradation of structurally important collagen molecules by inflammatory cell proteolytic enzymes. Using a rat model of acute myocardial infarction, significant collagen degradation was found to occur in the first twenty-four hours following experimental infarction. This degradation was prevented by eliminating inflammatory cells by prior whole body irradiation, or by treatment with a potent anti-inflammatory agent (ibuprofen). Continued treatment with ibuprofen resulted in enhancement of collagen deposition in the infarct wall.

PHS-6040
(Rev. 2-81)

Project Description: Collagen provides a structural network around heart muscle cells in normal myocardium. Following acute myocardial infarction (AMI) proteolysis of dead myocardium occurs associated with a massive influx of inflammatory cells. To determine whether collagen is also degraded (which might jeopardize infarct wall strength, leading to wall thinning or rupture), and if so, whether inflammatory cell proteases are implicated, we studied homogenized tissue from the normal zone (NZ), border zone (BZ), and ischemic zone (IZ) of control rats at 24 hours after AMI. This tissue was precipitated in trichloroacetic acid in order to separate large collagen molecules from proteolyzed fragments. NZ, BZ, and IZ was also obtained at 24 hours after AMI from rats made leukopenic (WBC less than 300/mm^2) by prior whole body radiation, and rats given ibuprofen 12.5 mm per kg at 6 and 18 hours after AMI. TCA-precipitated collagen was meaured as hydroxypvoline (nmole/mg/dry weight tissue; mean ±SE). (* =p<.001 vs control NZ).

	Control (n=5)	Leukopenic (n=5)	Ibuprofen (n=7)
N.Z.	9.0 ± 0.2	7.6 ± 1.6	9.6 ± 0.6
B.Z.	4.3 ± 0.5 *	8.0 ± 0.7	9.2 ± 0.7
I.Z.	5.8 ± 0.6 *	7.5 ± 0.4	8.5 ± 0.3

Thus, (1) within 24 hours after acute myocardial infarction there is significant degradation of large collagen molecules in the infarct region, especially in the border zone where the concentration of inflammatory cells is highest; and (2) collagen degradation is not seen in leukopenic rats or in rats treated with ibuprofen, a potent anti-inflammatory agent, indicating an important role of inflammatory cell proteases in early degradation of collagen after AMI.

To assess the effect of inflammatory cell proteases in the healing of the infarct region after AMI, tissue hemogenates from control rat IZ and NZ tissue was obtained at 1, 3, 7, or 21 days after AMI, and precipitated in trichloroacetic acid to separate intact from partially or completely degraded collagen. (*p<.001 vs. 1 day NZ, **=p<.001 vs. one day IZ).

	1d (n=5)	3d (n=5)	7d (n=10)	21d (n=6)
IZ	5.8 ± 0.6*	7.1 ± 1.2	69.0 ± 1.9**	96.0 ± 15.0**
NZ	9.0 ± 0.2	10.5 ± 1.8	16.0 ± 1.7*	17.8 ± 4.3*

Ibuprofen, 12.5 mg per kg, q12h was administered to rats beginning at 6 hours after AMI with sacrifice at 1,3, or 7 days. Another group received ibuprofen for 7 days with the sacrifice at 21 days (+ = p<.001 vs. respective control IZ) in preceeding table.

	1d (n=7)	3d (n=5)	7d (n=6)	21d (n=5)
IZ	8.5 ± 0.3 +	14.2 ± 0.6	107.5 ± 15.0+	188.0 ± 23.0+
NZ	9.6 ± 0.6	8.7 ± 0.7	13.6 ± 1.8	21.8 ± 1.8

Thus, continued administration of ibuprofen significantly enhanced collagen deposition, possibly by sparing newly synthesized collagen from continued inflammatory cell proteases. Future planned experiments will evaluate the morphologic and mechanical consequence of preventing the early degradation of collagen and enhancing collagen deposition in the infarct region following acute myocardial infarction.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 04016-01 CB

PERIOD COVERED: October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Regional Functional Properties of the Normal Dog Left Ventricle as Assessed by Gated Blood Pool Scintigraphy

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

PI:	Beverly Jones-Collins	Guest Worker	CB	NHLBI
Other:	Michael Green	Chief, Applied Physics Sec	NM	CC

COOPERATING UNITS (if any)

Nuclear Medicine, Clinical Center, NIH

LAB/BRANCH

Cardiology Branch, Bethesda, Maryland 20205

SECTION

Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION

NIHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.20	.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)

Heart function in normal dog hearts was analyzed in two different regions of the main pumping chamber (upper and lower poles) using a radioactive technique. Contraction and filling properties were not homogeneous. Rather, the lower pole contracted faster, ejected more of the blood in it and filled faster and earlier than the upper pole. This information is necessary to be able to analyze function in the different areas of an abnormal heart.

PHS-6040
(Rev. 2-81)

Project Description: Changes in the timing and magnitude of regional left ventricular (LV) volume variations in diseased hearts can only be assessed if regional variations in the normal heart are known. We studied regional volume variations in the control state in 10 dogs by gated blood pool scintigraphy In order identify the LV area supplied by the left anterior descending (LAD) coronary artery, we implanted balloon occluders around the proximal LAD. Gated blood pool scanning was performed before and after a two stage occlusion 19 days later. A normal and abnormal region of interest was selected from computer-generated maps (pixel by pixel) of Fourier phase and time to minimum volume for each dog in the post-occlusion state. These areas separated the LV area supplied by the LAD from the rest and were used to generate time activity curves in the pre-occluded state. The time to peak ejection rate and end systole was not different in the two areas, but the ejection fraction (73% vs 42%, $p<.005$), ejection rate (5.22 vs 3.37 edv/ sec, $p<.025$) and filling rate (5.98 vs 2.71 edv /sec, $p<.005$) were higher and time to peak filling rate (344 vs 325 msec, $p<.005$) was longer in the LV area supplied by the LAD. Significant functional variation therefore exists between the LAD-supplied area and the rest of the LV in normal dogs.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 04017-01 CB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
The effects of reperfusion and retrograde bleeding on the results of programmed right ventricular stimulation

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

PI:	B. A. Jones-Collins	Guest Worker	CB	NHLBI
Other:	Randolph Patterson	Senior Investigator	CB	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Cardiology Branch, Bethesda, Maryland 20205

SECTION
Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: .35	PROFESSIONAL: .30	OTHER: .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 ability of elecrical stimulation to provoke rhythm disturbances was tested 4 days after a heart attack in dogs. The heart attack was created in one of 3 ways: by blocking off a coronary artery permanently, by blocking off a coronary artery only temporarily, or by blocking off a coronary artery permanently and removing all blood flowing through the artery via collateral vessels for 2 hours. The group that had only a temporary coronary artery occlusion had far fewer provokable rhythm disturbances. This indicates that restoring patency of the coronary artery after a blockage has occurred may result in fewer rhythm disturbances.

PHS-6040
(Rev. 2-81)

Project Description: Reperfusion early after myocardial infarction is now being performed in patients (streptokinase infusion), but the effect on electrical instability of increasing or decreasing perfusion in the region at risk for myocardial infarction (RR) is unknown. 26 dogs were randomized to a control (9 dogs), reperfusion (10 dogs) or retrograde bleeding (7 dogs) group. All dogs underwent a coronary artery occlusion (17 of the left anterior descending and 9 of the circumflex artery). In control dogs the occlusion was permanent. In reperfusion dogs the coronary occlusion was released at 2 hours. In reperfusion dogs retrograde bleeding distal to coronary occlusion was continued for 2 hrs. Four days later all animals underwent a standard RV pacing protocol. Induced arrhythmias were scored and higher scores were assigned for: ventricular fibrillation>sustained ventricular tachycardia (VT)>non-sustained VT> repetitive ventricular response and for those provokable later in diastole. Myocardial infarction size was not different in the 3 groups (31%, 23%, and 35% of RR for control, reperfusion and retrograde bleeding. However, electrical instability index was much lower in the reperfusion than control or retrograde bleeding group (multivariate analysis, $p<.0001$ for reperfusion vs control, $p<.0018$ for reperfusion vs retrograde bleeding. Retrograde bleeding did not alter electrical instability from the control state. These results suggest that reperfusion post myocardial infarction may reduce electrical instability without changing myocardial infarction size.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 04018-01 CB

PERIOD COVERED October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
The Natural History of Electrical Instability Induced by Programmed Right Ventricular Stimulation after Myocardial Infarction in the Dog

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

PI:	B. A. Jones-Collins	Guest Worker	CB	NHLBI
Other:	Randolph Patterson	Senior Investigator	CB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Cardiology Branch, Bethesda, Maryland 20205

SECTION
Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.25	.20	.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 ability of electrical stimulation of the heart to provoke rhythm disturbances was tested 4 and 30 days after a heart attack in dogs. There were far fewer rhythm disturbances provokable at 30 than 4 days. This indicates a decrease in vulnerability of the heart to rhythm disturbances with the passage of time after a heart attack.

PHS-6040
(Rev. 2-81)

Project Description: Programmed right ventricular stimulation (PRVS) is often employed to evaluate electrical instability (EI) after myocardial infarction (MI). Spontaneous arrhythmic events are most frequent early after MI but it is not known how the results of PRVS change with time. Accordingly, 9 dogs underwent thoracotomy for implantation of a right ventricular (RV) pacing electrode and acute ligation of the left anterior descending (6 dogs) or circumflex (3 dogs) coronary artery. Four and 30 days later (for 5 survivors) a standard RV pacing protocol was carried out at twice diastolic threshold, a duration of 1.5 msec and a basic paced cycle length of 300 msec. The results of the protocol were scored to provide a numerical index of EI for each dog, for each test period. Higher scores were assigned to more hazardous ventricular arrhythmias [ventricular fibrillation>sustained ventricular tachycardia (VT)>nonsustained VT> repetitive ventricular response] and to those provokable later in diastole. EI index decreased from 4 days (61.3) to 30 days (.4) after MI ($p<.005$, paired t test). In summary, the results of PRVS in the canine model indicate considerable decrease in electrical instability between 4 and 30 days after MI.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 04019-01 CB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Regional Left Ventricular Function After Distal and Proximal Coronary Occlusion Measured by Gated Blood Pool Scintigraphy

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

PI:	Beverly Jones-Collins	Guest Worker	CB	NHLBI
Other:	Michael Green	Chief, Applied Physics Sec	NM	CC
	Randolph Patterson	Senior Investigator	CB	NHLBI
	Robert Miller	Radiotherapy Physicist	ROB	NCI

COOPERATING UNITS (if any)
Nuclear Medicine, Clinical Center, NIH

LAB/BRANCH
Cardiology Branch, Bethesda, Maryland 20205

SECTION
Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.20	.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)

Function in different areas (normal and abnormal) of the main pumping chamber of the heart was evaluated using a radioactive technique in dogs after a small and large heart attack. This technique was able to detect a decrease in function in the abnormal region (relative to its normal state) during the small heart attack and the large one. Thus, this technique can detect small heart attacks and can distinguish small from large ones.

PHS-6040
(Rev. 2-81)

Project Description: Left ventricular (LV) function measured by gated blood pool scintigraphy is depressed in patients with significant coronary artery disease. However, the ability of gated blood pool scanning to distinguish small from large ischemic regions is unknown. We studied 28 awake dogs, 18 days after thoracotomy to implant a left atrial catheter and 2 balloon occluders, proximal and distal on the left anterior descending (10 dogs) or circumflex (18 dogs) coronary artery. Gated blood pool scanning and microsphere measurements of myocardial blood flow (MBF) were performed in the control state, and after low (LO) and high occlusion (HO). Post-mortem dye staining measured the LO and HO ischemic areas (24 and 42% LV). Normal transmural blood flow existed in the LO and HO areas in the control state and decreased after occlusion (101 to 61% for LO and 96 to 34% of normal region MBF for HO). LO could be distinguished from HO on regional analysis of gated blood pool scanning: ejection fraction decreased from 48 to 31%; peak ejection rate (ER) from 3.39 to 2.25 edv/sec; time to ER increased from 129 to 150 msec; and peak filling rate (FR) decreased from 3.64 to 2.74 edv/sec. ($p<.02$ for all, LO vs HO). Time to FR did not distinguish between LO and HO. In summary, gated blood pool scanning can 1) detect small regions of myocardial ischemia and 2) distinguish small from large ischemic regions.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 04020-01 CB

PERIOD COVERED October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Verapamil-Induced Changes in Ventricular Volume and Diastolic Function in Hypertrophic Cardiomyopathy

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

PI:	R. O. Bonow	Senior Investigator	CB	NHLBI
Other:	H. G. Ostrow	Electronics Engineer	CS	DCRT
	D. R. Rosing	Senior Investigator	CB	NHLBI
	K. M. Kent	Head, Cardiovascular Diagnosis	CB	NHLBI
	L. C. Lipson	Senior Investigator	CB	NHLBI
	S. I. Allen	Medical Research Analyst	CS	DCRT
	S. L. Bacharach	Physicist	NM	CC
	M. V. Green	Chief, Applied Physics Section	NM	CC
	S. E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

Department of Nuclear Medicine, Clinical Center

Department of Computer Research and Technology

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: .11	PROFESSIONAL: .1	OTHER: .01

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Verapamil reduces left ventricular (LV) outflow tract gradients in many patients with hypertrophic cardiomyopathy. We studied the mechanism of this effect using a nonimaging ECG-gated scintillation-probe before and after intravenous verapamil. Despite reduction in blood pressure, verapamil increased LV end-diastolic volume, end-systolic-volume and stroke volume; increased peak LV filling rate; and decreased LV ejection fraction. This was associated with reduction in outflow gradient. Thus, verapamil reduces LV outflow gradient in pts with HCM presumably on the basis of increased LV volumes, which may relate to improved rapid diastolic filling.

PHS-6040
(Rev. 2-81)

Project Description: Verapamil (V) reduces left ventricular (LV) outflow tract gradients (OTG) and improves symptoms in many pts with obstructive hypertrophic cardiomyopathy (HCM). To investigate the relevant mechanisms for these effects, we studied 8 pts at catheterization with a nonimaging scintillation probe before (C) and after serial iv infusions of low, medium, and high dose verapamil (total dose 0.18, 0.45, and 0.80 mg/kg). Despite stepwise decreases in systemic blood pressure, verapamil increased LV end-diastolic volume (EDV) (C=100%, high dose verapamil=128±23% [±SD], $p < .01$) and end-systolic volume (C=100%, high dose verapamil=218±85%, $p < .01$), and decreased LV ejection fraction (C= 79±7, high dose verapamil=67±5%, $p < .01$). This was associated with a reduction in LVOTG (C=56±20, high dose verapamil= 12±12 mmHg, $p < .01$) in all pts and increase in stroke volume (SV) in 7 of 8 pts (C=100%, high dose verapamil=110±15%, $p < .05$). Peak LV filling rate increased (C=3.7±.9, high dose verapamil=4.7±1.1 EDV/sec, $p < .05$) after verapamil. Thus, high dose verapamil reduces LVOTG, presumably on the basis of increased LV volumes, which may relate to improved LV diastolic filling (DF). These data suggest that enhanced LV diastolic filling, reduced LVOTG, and increased SV contribute to the symptomatic improvement of many HCM pts during verapamil therapy.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 04021-01 CB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Scintillation Probe Measurement of Left Ventricular Diastolic Function in HCM

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

PI:	R. O. Bonow	Senior Investigator	CB	NHLBI
Other:	H. G. Ostrow	Electronics Engineer	CS	DCRT
	D. R. Rosing	Senior Investigator	CB	NHLBI
	L. C. Lipson	Senior Investigator	CB	NHLBI
	K. M. Kent	Head, Cardiovascular Diagnosis	CB	NHLBI
	S. I. Allen	Medical Research Analyst	CS	DCRT
	S. L. Bacharach	Physicist	NM	CC
	M. V. Green	Chief, Applied Physics Section	NM	CC
	S. E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)
Department of Nuclear Medicine, Clinical Center
Department of Computer Research and Technology

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.06	PROFESSIONAL: 0.05	OTHER: 0.01

CHECK APPROPRIATE BOX(ES)
☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Verapamil improves left ventricular (LV) diastolic filling in many pts with hypertrophic cardiomyopathy. To assess the effect of verapamil on simultaneous pressure volume (PV) relations, we generated high temporal resolution PV loops using a micromanometer catheter and a non-imaging ECG-gated scintillation probe in patients before and after intravenous verapamil. Preliminary data suggest that verapamil-induced improvement in rapid diastolic filling of the LV is associated with improved isovolumic relaxation and late diastolic PV relations.

PHS-6040
(Rev. 2-81)

Project Description: Verapamil (V) improves left ventricular (LV) diastolic filling, assessed noninvasively by radionuclide angiography, in many pts with hypertrophic cardiomyopathy (HCM). To determine whether these relative volume changes are associated with improved pressure-volume (PV) relations, we generated high temporal resolution PV loops using micromanometer catheters and a nonimaging ECG-gated scintillation probe in 5 pts before (C) and after intravenous verapamil (total dose 0.4-0.8 mg/kg). Heart rate was maintained by atrial pacing. After verapamil, LV end-diastolic volume (EDV) increased in all pts (C=100%, V=122±6% [±SD], $p<.05$). Peak filling rate (PFR) increased in 3 pts (C=3.4 ±1.3, V=4.4±.8 EDV/sec), and in all 3 pts LV end-diastolic pressure (EDP) decreased (C=20 ±9, V=15±8mmHg), the time constant (T) of LV relaxation decreased (C=58±20, V=38± 5 msec), and the PV curve was shifted downward and rightward throughout diastole. In contrast, in the 2 pts with no improvement in PFR (C=4.7± .1, V=4.4±.2 EDV/sec,), LVEDP did not change (C=15±6, V=15±6 mmHg) and T increased (C=53±12, V=69±18 msec). Thus, improved LV rapid diastolic filling induced by verapamil in a subgroup of pts with HCM is associated with improved isovolumic relaxation and late diastolic PV relations.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 04022-01 CB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Hemodynamic Effects of Nifedipine in Patients with Hypertrophic Cardiomyopathy

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

PI:	D. R. Rosing	Senior Investigator	CB	NHLBI
Other:	R. O. Cannon	Clinical Associate	CB	NHLBI
	K. M. Kent	Head, Cardiovascular Diag.	CB	NHLBI
	L. C. Lipson	Senior Investigator	CB	NHLBI
	R. O. Bonow	Senior Investigator	CB	NHLBI
	H. G. Ostrow	Electronics Engineer	CS	DCRT
	S. E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

Department of Computer Research and Technology

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: .1	PROFESSIONAL: .05	OTHER: .05

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Verapamil, a calcium channel blocking agent, has been shown to have beneficial hemodynamic effects in patients with hypertrophic cardiomyopathy. To assess whether other drugs of this type have similar effects, nifedipine was administered buccally in the Catheterization Laboratory to patients with this disorder. Although nifedipine appeared to decrease left ventricular outflow obstruction, its lack of effect on left ventricular systolic pressure and early diastolic filling raises questions as to whether it will be as effective as verapamil.

PHS-6040
(Rev. 2-81)

Project Description: Verapamil (V) exerts beneficial hemodynamic and clinical effects in patients with hypertrophic cardiomyopathy (HCM). To assess whether nifedipine (N) has similar beneficial hemodynamic properties, N was given buccally to 20 HCM pts at the time of catheterization. Heart rate (HR), systolic blood pressure (SBP), mean pulmonary wedge pressure (PCW), cardiac index (CI), and left ventricular (LV) outflow tract gradients (GRAD) were measured before (C) and after a total of 10-30 mg of N ($=p<0.001$). Peak effects were:

	HR	SBP	PCW	CI	GRAD
C	77±13	127±15	17±9	2.9±0.9	54±36
N	91±14	111±15	17±9	3.6±0.9	60±42

The lack of change in LV GRAD when CI increased indicates a reduction in LV outflow tract obstruction or increase in effective LV outflow orifice area, although LV systolic pressure did not decrease. Moreover, in contrast to prior rest radionuclide studies with V, which was shown to LV peak filling rate (PFR) and time to peak filling rate (TPFR), PFR and TPFR were unchanged by N. Thus, although N increases CI in pts with HCM its lack of effect on LV systolic pressure and early diastolic filling raises questions as to whether it will be as effective as V in symptomatic control of pts with HCM.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 04023-01 CB

PERIOD COVERED October 1, 1980 to July 30, 1981

TITLE OF PROJECT (80 characters or less)
Validation of Intramyocardial Fiberoptic pH Probe for Use in the Study of Myocardial Ischemia

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

PI:	R. M. Watson	Clinical Associate	CB	NHLBI
Other:	R. E. Patterson	Senior Investigator	CB	NHLBI
	D. R. Markle	Investigator	R	BEI
	D. A. McGuire	Investigator	R	BEI
	S. R. Goldstein	Chief, Mechanical Engineering Sec.	R	BEI
	J. I. Peterson	Chief, Chemical Engineering Section	R	BEI
	S. E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)
Division of Research Services, Biomedical Engineering and Instrumentation Branch

LAB/BRANCH
Cardiology Branch

SECTION
Experimental Physiology and Clinical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 2 1/2	PROFESSIONAL: 1 1/2	OTHER: 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 unique fiberoptic pH probe was designed by the Biomedical Engineering and Instrumentation Branch which permits monitoring of intramyocardial pH. Studies are in progress to demonstrate that this probe reflects true pH values as compared to accepted techniques. The probe has been shown to read known buffers and arterial blood gas samples as confirmed by an IL, Instrumentation Laboratory pH/Blood Gas Analyzer. The stability of the pH readings over hours has been demonstrated. The implanted probe records a normal pH of 7.4 prior to partial coronary artery occlusion in open chest anesthetized dogs. Intramyocardial pH falls with ischemia and recovers quickly with reperfusion. Metabolic and respiratory and base changes are effected and reflected by the intramyocardial pH probe. A pH gradient across the heart muscle wall has also been demonstrated.

PHS-6040
(Rev. 2-81)

Project Description: Experiments were performed in open chest anesthetized dogs to refine and validate a miniature fiberoptic pH probe developed for physiologic use. Initial difficulties with intramyocardial implantation and stable probe position maintenance prompted several modifications with the ultimate development of a second generation probe.

The probe was shown to read accurately known buffers both prior to and after eight hours of implantation in open chest dogs. Stability of the probe readings over hours has been documented. Likewise, the probe correctly reads the pH of arterial blood gas samples as confirmed by the IL, Instrumentation Laboratories pH/Blood Gas Analyzer.

Studies are in progress during intramyocardial implantation which show:

(1) a normal intramyocardial pH prior to coronary artery occlusion.
(2) a fall in intramyocardial pH with partial coronary artery occlusion.
(3) a further fall in intramyocardial pH with total coronary artery occlusion.
(4) a rise in intramyocardial pH with reperfusion of the ischemic area.
(5) a fall in intramyocardial pH with hypotension (decreased coronary perfusion pressure).
(6) a fall in intramyocardial pH with VT/VF
(7) a fall in intramyocardial pH, reflecting arterial blood pH with creation of metabolic and respiratory acidosis.
(8) a rise in intramyocardial pH, reflecting arterial blood pH with creation of metabolic or respiratory alkalosis.
(9) a fall in intramyocardial pH of the ischemic zone with pacing.

Studies are in progress to correlate intramyocardial pH with myocardial blood flow as determined by radioactive tracer microspheres. A transmural pH gradient in the ischemic zone has been documented.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 04024-01 CB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Side Effects of Oral Verapamil Therapy in Patients with Cardiac Disease

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

PI:	D. R. Rosing	Senior Investigator	CB	NHLBI
and	Terry Rehder	Pharm. D.	Pharm	CC
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COOPERATING UNITS (if any)

Department of Pharmacy

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.1	.05	.05

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Verapamil administration has resulted in occasional life threatening side effects related to its physiological actions. A number of less serious complaints have also been elicited in patients, but these "adverse effects" have rarely either limited the dosage of the drug which could be administered or prevented its continued use. A protocol was developed in order to determine the frequency, significance, and relationship to verapamil administration of these "adverse effects". Early results indicate that the drug appears to be well tolerated, but a few unsuspected potential side effects may have been uncovered.

PHS-6040
(Rev. 2-81)

Project Description: Verapamil has been characterized as a well tolerated, safe drug in the cardiologic literature. Most of the reports regarding the type and incidence of side effects from this drug have originated from studies performed outside the United States. During our initial experience with verapamil, we became aware of the potential life threatening adverse effects which could occur as a result of the physiological effects of verapamil--negative inotropy, peripheral vasodilatation, conduction system depression. Less obvious was the capacity of this agent to produce less serious side effects.

In order to determine the frequency and significance of non-life threatening side effects, a patient questionaire was designed. At the present time, 42 patients on chronic verapamil therapy have been interviewed. When it became apparent that the list of possible side effects would be legion, the questionaire was also administered to patients on beta adrenergic blocking drugs for comparison. Eventually, patients on no cardiac drugs will also be interviewed. The demographic data obtained include:

			Disease		Drug	
Sex		Age (X)	CAD	HCM	Verapamil	Propranolol
Women	15	49 Y/O	2	13	14	1 (CAD)
Men	35	50 Y/O	16	19	28	7 (IHCM)

Side effects of the two medicines are compared in the attached table. The major side effects definitely related to verapamil administration appear to be constipation, arthralgias, and weight gain. In patients on propranolol, frequent adverse effects included depression, memory loss, worsening of temperament, decreased libido fatigue, and urinary urgency. Only five of over 20 patients started on verapamil have been unable to tolerate the drug. The reasons for discontinuing its use were upper gastrointestinal discomfort, rash, nausea and vomiting (2) and memory loss.

In summary, although minor side effects are common verapamil appears to be a well-tolerated drug and few patients have had to discontinue its use or modify their dosage because of non-life threatening drug side effects.

Publications: None

Annual Report of the
Laboratory of Cell Biology
National Heart, Lung and Blood Institute
October 1, 1980 to September 30, 1981

In the summary of this year's activities of the Laboratory of Cell Biology, emphasis will be given to research in three related areas: (1) the biochemistry of muscle contraction, (2) the polymerization of actin, and (3) regulation of myosins from non-muscle cells.

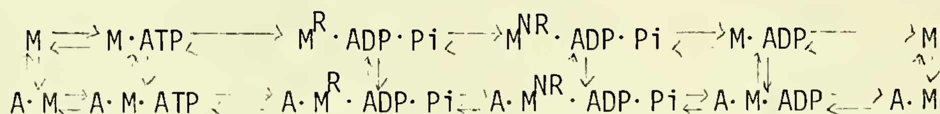
(1) Biochemistry of Muscle Contraction: Muscle contracts as a result of the interaction of thin and thick filaments. In brief, the contractile unit (the sarcomere) consists of two structures (the Z-bands) from which attached parallel thin filaments project towards the center of the sarcomere. Thick filaments, which are not attached to the Z-bands, interdigitate between the thin filaments. The thick filaments are polymers of multiple protomers of actin, a globular protein of molecular weight 42,000, and associated regulatory proteins, tropomyosin and troponins. The thick filaments consist of myosin protomers, a molecule of molecular weight about 500,000 containing two heavy chains (200,000) and two pairs of light chains. The bipolar thick filament is formed by association of the double helical rod portions of the myosin heavy chains; the globular heads of the heavy chains, with their associated light chains, project from the thick filaments. The heads have opposite orientation at the two ends of the thick filaments. Driven by the hydrolysis of ATP, myosin heads attach to the actin filaments at an angle of about 45°, then rotate to an angle of about 90°, detach and repeat the cycle. The bipolarity of the myosin thick filaments results in the actin filaments that project from the opposite Z-bands being pulled towards each other and, thus, the sarcomere shortens as the thin filaments slide past the thick filaments. The prevailing idea has been that re-binding of ATP dissociates the myosin heads from the actin filaments and that the two sets of filaments then re-associate and translocate, as described, as the ATP is hydrolyzed to ADP and Pi. Thus, the chemical energy released by the ATPase cycle is coupled to the crossbridge cycle to produce movement.

Concepts of the mechanism of the transduction of the chemical energy of ATP hydrolysis into mechanical work have been greatly modified as the result of investigations under the direction of Dr. Evan Eisenberg. In the hypothesis that he has developed, in collaboration with Dr. Terrell Hill, NIADDK, myosin is conceived as having the possibility of existing in a 90° state and a 45° state, i.e. in conformations that have their lowest energy when the head is at an angle of 90° or 45°, respectively, with respect to the filament. But myosin in either conformational state can exist at any physical angle.

Moreover, experimental evidence suggests strongly that actin and myosin are essentially always attached to each other during the ATPase cycle. When the myosin contains either bound ATP or ADP·Pi, the actin-myosin attachment is relatively weak; when the myosin contains bound ADP, the actin-myosin attachment is relatively strong. Therefore, as ATP is hydrolyzed by a particular myosin head, that head will transform from the 45° state to the 90° state. When a head in the 90° state is at an angle of 45°, the crossbridge will be under tension which allows work to be done (the actin filament to be moved) as the attached head rotates to a 90° angle. As the attached head moves past the 90° angle (caused by the actin filament being moved by other myosin heads) it is stretched and now exerts tension in the opposite, counter-productive direction. But, by

this time, ATP can reassociate with the myosin head, displacing ADP, and weaken the attachment between the myosin and actin. The myosin head will rapidly dissociate from the actin filament and then re-attach at its now preferred angle of 45°.

Thus, instead of the ATPase cycle occurring with the myosin associated with the actin during part of the cycle and dissociated during other parts of the cycle, as previously thought, according to this new concept the myosin will be attached to actin essentially throughout the entire cycle. The scheme of the proposed molecular events is as follows:



States M·ATP and M·ADP·Pi would be strongly attached to actin and states M·ADP and M would be weakly attached to actin. The model still includes the previously proposed transition of M^R·ADP·Pi to M^{NR}·ADP·Pi (i.e. a refractory state of myosin to a non-refractory state of myosin) as a necessary step between the hydrolysis of ATP and release of Pi. This step was originally proposed as a transition of myosin from refractory state that was unable to bind to actin to a non-refractory state that could bind to actin. The proposed conformational change in myosin has been retained to explain how maximal actomyosin ATPase activity can occur even when all the myosin is not bound to actin. A slow, rate-determining transition of A·M^R·ADP·Pi to A·M^{NR}·ADP·Pi is sufficient explanation. The only alternative, if that transition were not to be proposed, would be for the hydrolysis step, A·M·ATP to A·M·ADP·Pi to be rate-limiting in the presence of excess actin and for the release of Pi to be very rapid. If this were so, the concentration of myosin-bound ADP Pi (the Pi burst) would necessarily decrease as the concentration of actin was increased. New experiments have shown this not to be the case and, therefore, it seems quite likely that the above model, including the refractory and non-refractory states, is correct.

Contraction of skeletal muscle is regulated by the interaction of Ca^{2+} with the complex of tropomyosin and troponins that is associated with the actin filament. The biochemical correlate of this physiological event is that tropomyosin-troponin inhibits actomyosin ATPase activity in the absence of Ca^{2+} and enzymatic activity is restored upn addition of Ca^{2+}. The traditional interpretation of this process is epitomized as the steric-blocking model which assumes that myosin is sterically blocked from its functional interaction with actin by the presence of tropomyosin-troponin on the actin filament in the absence of Ca^{2+} and that Ca^{2+} causes a shift of the tropomyosin-troponin to a non-blocking position.

Evidence against this model was obtained several years ago in this Laboratory, when it was observed that tropomyosin activated, rather than inhibited, actomyosin ATPase activity when Acanthamoeba actin was substituted for muscle actin. Moreover, when a random copolymer of Acanthamoeba and muscle actins was used, tropomyosin activated the Acanthamoeba actin-myosin ATPase activity and inhibited the muscle actin-myosin ATPase activity in the same filament. Such data are difficult to reconcile with a steric model for the action of tropomyosin.

Last year, it was observed that myosin ADP bound cooperatively to F-actin filaments in the presence of tropomyosin-troponin (regulated actin) but independently to actin in the absence of tropomyosin-troponin (unregulated actin). The cooperative binding was analyzed into two components: the equilibrium constant between weakly and strongly binding forms of actin and the interaction between tropomyosin molecules. According to this model, the binding of myosin to regulated actin should not change when other nucleotides are substituted for ADP. New experimental data, however, show that the nature of the nucleotides bound to the myosin does modulate the cooperativity of the myosin binding although, as expected, the cooperativity _per se_ remains as a property of the actin filament.

Thus, in the absence of Ca^{2+}, tropomyosin-troponin inhibits the binding of myosin and actin less when AMP PNP is the nucleotide than when ADP is the nucleotide or when there is no bound nucleotide. Little effect of nucleotide is seen in the presence of Ca^{2+}. With or without Ca^{2+}, at saturation, more myosin is bound to regulated actin than to unregulated actin. This potentiation of myosin binding to actin filaments is also affected by the nucleotide. These, and other, data suggest that myosin binds to actin in different conformations in the presence of ATP and AMP·PNP which leads to the following model for the effect of tropomyosin-troponin on the actomyosin ATPase.

Tropomyosin-troponin might inhibit hydrolysis in the absence of Ca^{2+} not by blocking the binding of myosin·ATP to the actin filament but by inhibiting the release of Pi from the actomyosin ADP·Pi complex because of the much weaker affinity of actin for myosin·ADP than for myosin ATP. This is equivalent to hindering rotation of the myosin head from the 90° state to the 45° state.

This interpretation, which is an alternative to the steric-blocking model, is compatible with other data obtained in this Laboratory this year by comparing the binding of myosin·ATP to regulated actin with the ATPase activity, in the presence and absence of Ca^{2+}. The ATPase activity was more than 20 times greater in the presence of Ca^{2+} but the association constant of myosin ATP for actin was unaffected by Ca^{2+}. These results suggest that tropomyosin-troponin regulates a step in the ATPase cycle and not the binding of myosin to actin.

(2) The Polymerization of Actin: Actin is essential, as described in the previous section, for the contraction of muscle but it is also essential for the maintenance of the organization of non-muscle cells and for the movement of non-muscle cells during such activities as cell division, phagocytosis and clot retraction. In contrast to the situation in muscle cells, more than half of the actin in non-muscle cells exists in the non-polymerized state and the regulation of the state of polymerization of actin is a major control mechanism for many physiological processes. In the polymerization of actin, one molecule of ATP is hydrolyzed for every molecule of actin that is converted from the monomeric to the polymeric state. However, the energy of hydrolysis of ATP is not utilized in any way to perform work and actin can polymerize essentially normally when ADP or non-hydrolyzable analogues of ATP are substituted for ATP. It is very expensive for the cell to utilize so much ATP in this way and, therefore, it becomes important to understand the function of ATP hydrolysis in the polymerization process. To do this, it is necessary to understand the mechanism of ATP hyddrolysis.

Investigations under the direction of Dr. Korn have found that monomeric actin has a significant but low ATPase activity that can be greatly stimulated by the additions of any one of four types of cytochalasins. The minimal steps in an actin-catalyzed ATPase cycle must include the following:

Actin·ATP ⇌ Actin·ADP·Pi → Actin·ADP ⇌ Actin ⇌ Actin·ATP

in which actin-bound ATP is hydrolyzed to the actin-bound products ADP and Pi which are then released sequentially (first Pi and then ADP) followed by the re-binding of ATP. There may, by analogy to other ATPases, be conformational intermediates before and/or after the formation of actin ADP·Pi. Quantitative kinetic analysis of the cytochalasin-stimulated ATPase activity of monomeric actin led to the conclusion that the rate limiting step in the absence of cytochalasin is the conversion of actin ADP·Pi to actin·ADP + Pi and that this step is accelerated by the formation of a cytochalasin actin ATP to actin ADP Pi complex until the rate-limiting step becomes the rate of conversion of actin ATP to actin ADP·Pi. Since this hydrolysis occurs maximally under ionic conditions favorable to polymerization, all the species in the ATPase cycle will necessarily be present during polymerization and any one of them might be the preferred species for polymerization. If the preferred species were not actin ATP, for example if the preferred species for polymerization were actin ADP·Pi, kinetic regulation of the ATPase cycle could provide a sensitive cellular mechanism for the regulation of actin polymerization.

Attempts to measure directly the existence of actin-bound ADP·Pi during the monomeric actin ATPase cycle have been unsuccessful. This is not unexpected since the equilibrium in the hydrolysis step, actin·ATP ⇌ Actin·ADP·Pi, would be expected to lie far to the right in favor of actin·ATP. For various theoretical reasons, and from experimental observations, moreover, it seemed necessary that thehydrolysis step, or at least the release of Pi, would occur from the actin polymer, i.e. after the addition of the monomeric actin to the end of the filament. This is necessary to propose because if the hydrolysis occurred before addition of the actin monomer to the polymer end there would be no difference between the polymeriation of actin·ADP and actin·ATP. In essence, this proposal means that the hydrolysis of ATP that accompanies polymerization would occur with part of the ATPase cycle occurring on monomeric actin and the remainder of the ATPase cycle after the actin molecule because of the terminal actin protomer of the elongating filament.

To determine the validity of this hypothesis, one would like to be able to analyze for actin-bound ADP·Pi on the actin polymer. This is essentially impossible to do, however, because the filaments are very long and most of the actin subunits would have bound ADP. The filament ends, which would be the place to expect the bound ADP·Pi, are present in too low a percentage of the total actin in the polymer to make the analysis possible. Therefore, we prepared chemically covalently crosslinked actin dimers from actin polymers in the hope that the dimers would retain some of the properties of the polymeric actin from which they were derived. In fact, some evidence has been obtained that the actin dimer has a conformation somewhat intermediate between monomeric G-actin and polymeric F-actin. The actin dimer binds, as expected, to moles of nucleotide and, like monomer, binds ATP more tightly than ADP. One of the dimer ATP sites exchanges more rapidly than the other and this more rapidly exchanging site seems to contain about 15% of its nucleotide as the bound products ADP·Pi. Thus, these data provide some experimental support for the concepts that the crosslinked

dimer may be a useful model for filament ends, that filament ends may contain bound ADP·Pi and that actin·ADP·Pi may be at least one of the monomeric species involved in the polymerization of actin.

In cells, the concentration of unpolymerized actin is much greater than can be explained by the equilibrium between monomeric and polymeric actin under equivalent conditions working with pure actin in the test tube. One reason for this is the existence of a protein, called profilin, that forms a 1:1 molar complex with monomeric actin and inhibits its polymerization. Previous indirect analysis had led to the conclusion that the K_D for the interaction of profilin and actin was about 5 X 10^{-5}M. Recent direct binding studies have lowered that value to about 5 X 10^{-6}M which is much more compatible with the role of profilin in inhibiting polymerization. Previous results had emphasized that profilin inhibited the first step of polymerization, the nucleation reaction in which 3 or 4 actin monomers form a trimer or tetramer which then can rapidly elongate. The newer data confirm this but show that profilin also affects the final steady state concentration of actin polymer, as it should. In the absence of a molecule such as profilin a very great difference in intracellular conditions would be required to change the steady state distribution of actin between monomeric and polymeric state. The steady state concentration of filamentous actin, in the absence of profilin, would be:

$$[A_F] = [A_t] - [A]_\infty$$

where Af = filamentous actin, A_t = total actin and [A] = critical concentration of monomeric actin in equilibrium with filamentous actin. In the presence of profilin, the expression would be modified as follows:

$$[A_f] = [A_t] - [A]_\infty - ([P_t]\,[A]_\infty)/([A]_\infty + K_D)$$

where Pt = total profilin and K_D = dissociation constant for the interaction of profilin and actin monomer. As a result, a small change in ionic conditions that leads to a small change in $[A]_\infty$ would have a much greater effect on $[A_F]$ in the presence of profilin than in its absence.

(3) Regulations of Myosin from Non-Muscle Cells: The actomyosin ATPase activity of skeletal muscle that provides the energy for the contractile events, is regulated by the interaction of Ca^{2+} with the complex of tropomyosin-troponin that is associated with the actin thin filaments. In contrast, the actomyosin ATPase activity of smooth muscle is regulated, at least in part, by the Ca^{2+}-dependent phosphorylation of one of the light chains of the myosin. The phosphorylated form of the myosin is enzymatically active, the dephosphorylated form is enzymatically inactive. There is also evidence that myosins of mammalian non-muscle cells are similarly regulated through the phosphorylation of their light chains. On the other hand, a novel mechanism of regulations of the myosin isoenzymes of Acanthamoeba has been discovered in this Laboratory which may be applicable to other species but, even if not, promises to shed considerable light on the mechanism of the actin-activated ATPase reaction of myosin.

Acanthamoeba has previously been shown to contain three myosin isoenzymes. Two of these, myosins IA and IB, are unusul in having only one hevy chain and one each of two kinds of light chains. They are also unusual in that their actin-activated ATPase activities are regulated by phosphorylation of their heavy chains, rather than phosphorylation of a light chain. The phosphorylated forms

of myosins IA and IB are the enzymatically active forms. A third myosin isoenzyme, myosin II, has a pair of heavy chains and two pairs of light chains. It too, however, has previously been shown to be regulated by phosphorylation of its heavy chains but in this unique case the phosphorylated form of the enzyme is the enzymatically inactive form and the dephosphorylated myowin is enzymatically active.

This year we have found that there are, in fact, three phosphorylation sites on each of the heavy chains of myosin II which are phosphorylated in parallel by a partially purified heavy chain kinase. Phosphorylation converts an active enzyme to an inactive enzyme. Moreover, all three phosphorylation sites on each heavy chain have been found to be within a small segment no larger than about 3000 daltons at, or very close to, the carboxyl-terminal end of the molecule. The ATPase site is at least 100,000 daltons away in linear sequence, separated from the phosphorylation site by the rigid double helical rod portion of the myosin molecule. It then presents quite an interesting challenge to determine how a regulatory site can effect a catalytic site from which it is so distantly removed. One possibility is that a regulation of ATPase activity occurs through the interaction of two or more myosin molecules with each other. Some evidence has been obtained for an increase in specific activity as a function of myosin concentration which implies a myosin-myosin interaction. It is likely that much of the myosin in non-muscle cells is non-filamentous and that filament formation as well as enzymatic activity is under active regulation. It would be reasonable if the state of phosphorylation of myosins concomitantly regulated myosin-myosin interction, perhaps to form filaments, and myosin ATPase activity.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00401-15 LCB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Potentiometric studies of respiratory components of E. coli and rat liver

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:	Richard I. Shrager	Mathematician	LSMM DCRT
	Hideo Kon	Physical Chemist	LCP NIAMD

COOPERATING UNITS (if any)

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Laboratory of Chemical Physics, NIAMD

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Section on Membrane Enzymology

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 0.8	PROFESSIONAL: 0.8	OTHER: 0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The membrane-bound enzyme system which causes acidification of the medium upon oxidation through a critical voltage range above 300 mV has been found to operate in beef heart mitochondria. An essential component of the reducing power in the E. coli system has been identified as a charge transfer complex involving an amine and a quinone. The source of protons has been identified as water. A powerful new technique for resolving a complex series of spectra into individual spectra and the quantitative and thermodynamic properties of the individual components such as redox potentials, number of electrons transferred in an oxidation and pK's for acids has been developed and tested.

PHS-6040
(Rev. 2-81)

Background: We have described a system which causes the release of acid when *E. coli* membranes or a detergent extract of the membranes is oxidized through a narrow voltage range of ≃320 to ≃335 mV. The system appears to involve a high potential iron protein.

Major findings: We have determined that the same system appears to operate in beef heart mitochondria although the mammalian organelles require the presence of deoxycholate in order to demonstrate its activity. All further developments described in this report relate to the bacterial system.

Copper is not required for the operation of the membrane-bound system, but the detergent-extracted system operates most efficiently in the presence of ~0.06 mM Cu^{2+}. We have determined that Cu^{2+} is needed not only for acid production but also for development of the characteristic EPR signal which arises during the process of acid liberation.

The two most fundamental and mysterious aspects of the system are the source of electrons consumed during the electric oxidation and the mechanism of the acid production. With the realization that Tris buffer which contains three primary hydroxyl and one amino group could be an electron donor, we proceeded to explore this possibility. Omitting Tris did prevent the acidification and a quantitative assay for carbonyl groups based on 2,4 dinito-phenylhydrazone did show an increase in carbonyl groups during oxidation in the presence of Tris. In order to determine what parts of the Tris molecule were being oxidized and in the hope of finding a less complex electron donor, various analogues of Tris were tested. 2-Aminopropanol which has only one primary hydroxyl and one primary amino group caused about 1/2 the acid production as did Tris, but n-propanol having no amino group was inactive. On the other hand, L-alanine having only the primary amino group and no primary alcohol, was as active as amino propanol. Further studies with D-alanine, N-methyl-L-alanine, and proline confirm that it is only the amino group which is important and that a secondary amine is about twice as active as a primary amine. With the secondary amines, a unique optical spectrum and deep magenta color develops just prior to acid production. In the voltage range for acid release, there is a progressive loss of the magenta color as acid is formed. The magenta-colored compound is formed from a secondary amine plus benzoquinone or catechol. The compound responsible for the color is a "chage transfer complex" in which one of the unbonded pair of electrons from nitrogen is held by the quinone ring structure. If the *E. coli* extract is omitted from the system, electric oxidation produces no acid, the magenta color does not disappear and the optical spectrum attributed to the high potential iron protein does not form. Similarly if the secondary amine and *E. coli* extract are present but the quinone is omitted, no reaction takes place. With the complete system, acidification ceases when only 10% of the amino compound has been consumed as determined with the amino acid analyser. It is significant that the total molar amount of quinone present is ~10% of that of the amino compound. Using ^{14}C-proline as the secondary amine, the 10% of oxidized proline is seen to be present in two major products which are eluted very early from the resin column.

The other major aspect of the problem requiring elucidation is the source and mechanism of liberation of protons. The experimental approach used to attack this problem has been based on the realization that none of the particles or solutes present has sufficient protons to account for the magnitude of acid released except for the phosphate. The proof that phosphate provides the protons requires that we identify the location or nature of the phosphate after it has been stripped of protons. To this end, we have employed centrifugation, gel

chromatography, ion exchange chromatography, ^{31}p-NMR both for soluble and "solid state" sources, and thin layer chromatography. No sequestered pool of alkaline phosphate or new chemical form of phosphate has been found. We therefore must reject the idea that phosphate is the source of the protons although phosphate does appear to participate in the acid liberation phenomenon. The only remaining source for the protons is water. There is a membrane-associated enzyme which uses an amino-quinone complex to mobilize H·atoms from water and allow their oxidation to H+.

Resolution of Complex spectra: A very important problem that we have faced in our work is the resolution of a large series of optical spectra in terms of individual spectral components all of which respond independently to voltage or pH according to a known law such as Nernst or Henderson-Hasselbach. We have previously developed techniques based on changes in the 2nd derivatives of absorbance *vs* wavelength at peak absorbance and on Gaussian shapes of absorbance peaks. Although these procedures have been successful, they are not ideal in that some subjectivity can enter into the modeling and complete optical spectra are not obtained. We have therefore sought and have been successful in developing a new powerful technique based on a mathematical technique called singular value composition. The input to the procedure is a large matrix made up of individual spectra of a mixture of components under different conditions of pH or voltage. The final output is a collection of complete individual spectra and parameters for each, such as amounts, pK's and/or redox potentials. The method was proved using a test mixture of three pH indicators with similarities in optical spectra and fairly close pK's and with the mixture of respiratory pigments of *E.coli* membranes previously analyzed by the Gaussian technique. The new method should be of use to other workers confronted with analysis of complex spectra of any type.

Proposed Course of Project: Attention will be centered on establishing the details of the process by which protons can be liberated from water by a membrane-bound enzyme using an amino-quinone complex. We will try to understand the significance of such an enzymatic activity in terms of biological function. If a suitably rapid assay technique can be developed, we will try to isolate the enzyme involved.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00402-09 LCB

PERIOD COVERED
October 1, 1980 to September 30, 1981

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

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Other:	Raymond Scharff	Physiologist	LCB NHLBI
	Musetta Hanson	Staff Fellow	LCB NHLBI

COOPERATING UNITS (if any)

NONE

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Section on Membrane Enzymology

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.7	1.7	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)
Further proof was obtained for the actual incorporation of DNA polymerase I into a complex of higher molecular weight which unlike the free polymerase, is active with native DNA templates. It was shown that the loss of 5'→3' exonuclease from DNA polymerase I renders the molecule less hydrophobic as well as making it incompetent for complex formation. This plus the genetic evidence which shows that the 5' → 3' exonuclease is required for cell viability plus the fact that a factor "E", essential for complex formation and stability, is associated with the membranes suggests that complex may perform some essential function at the cell membrane surface.

PHS-6040
(Rev. 2-81)

Major findings: A membrane-associated component, "E", has been described in the past two reports. This component can be released from the membrane by deoxycholate. "E" preserves the native DNA-polymerizing complex from being dissociated by dilution, apparently by shifting the equilibrium in favor of the complex. Crude "E" converts free DNA polymerase I to complex. A trivial explanation of the observed effects of "E" is that "E" is a DNase which causes DNA polymerase I to accept native DNA by introducing "nicks" into the duplex structure thus activating it for the polymerase. The same reasoning could explain complex formation as only apparent if "E" migrates slower than DNA polymerase I in the acrylamide gel and activates only some tailing polymerase which overlaps the DNase. To test these ideas, a sensitive technique for detecting DNase activity in gels was developed. "E" showed no DNase activity. In another approach, DNase I, which is capable of activating native DNA, was diluted to a level just below the threshold for detection in the gel. DNase I migrates to the same Rf or just a bit slower than polymerase I. The DNase at that level of activity did not render the polymerase detectable in the assay systems using native DNA, which does detect the activity of the complex in the gel. Therefore, the trivial explanation is unsupported by direct tests. Positive support for the concept that polymerase I is physically incorporated into a complex is the demonstration that when "E" is added in increasing quantities to polymerase I, the PAGE spot of polymerase I disappears as the new spot for complex appears. Furthermore, when isolated complex is diluted, the spot for complex disappears and the spot for polymerase appears. The previous report showed that commercial polymerase I produced two active spots in PAGE but that only the slower one could be converted to complex by "E" and that only the slower spot was released by complex upon dilution. We have identified the faster spot as the Klenow fragment which is derived from polymerase I by a single hydrolytic split which removes the 5'→3' exonuclease activity. It is of interest that this fragment must be present in DNA polymerase I for cell viability and for the formation of the complex. Another interesting finding is that when Triton and deoxycholate are added to the PAGE system, the intact DNA polymerase is greatly retarded in its migration, whereas the Klenow fragment is much less affected. This indicates a higher degree of hydrophobicity for the complete polymerase molecule compared to the molecule missing the vital 5'→3' exonuclease peptide. This plus the memebrane localization of "E" suggests the hypothesis that complex may naturally be formed at a membrane surface.

Much of the work of this past year has been the repeating of the basic observations discussed in this and earlier reports in preparation for writing a manuscript for publication.

Proposed course: So far, attempts to purify "E" and complex have not been successful. This has been due to the loss of activities during purification. Different techniques will be tried to achieve these needed isolations.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00405-07 LCB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Circular Dichroic Studies of Protein Denaturation

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

PI: Frederick H. White, Jr. Research Chemist LCB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Section on Cellular Biochemistry and Ultrastructure

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) It is well known that development of conformational structure is dependent on amino acid sequence. However, the exact relationship, which would make possible the reliable production of native conformation from a knowledge of primary structure, remains unclear. Toward this goal, we have been concerned with the role of SS bonds in the formation and maintenance of native conformational structure. Conditions have been developed for the partial and complete reduction of lysozyme, under non-denaturing conditions, such that any observed structural changes would result specifically from SS cleavage. Intermediates representing all possible stages of reduction have been isolated from partially reduced, carboxymethylated samples of lysozyme by ion exchange chromatography. The CD characteristics of these intermediates indicate a high level of cooperativity between SS bonds and secondary structure, since they resemble those of the fully reduced protein throughout all stages of reduction. The presence of intermediates during reduction, however, contrasts with literature reports for some other proteins, according to which all measured elements of structure have disappeared or reappeared simultaneously. The present findings are consistent with formation of a critical SS bond content prior to a major secondary structural transition.

Objective: To examine conformational structures remaining after SS bond cleavage as they relate to the folding process.

Methods:

(1) Original methods have been elaborated for both partial and complete reduction of lysozyme in the absence of denaturant.
(2) Circular dichroic (CD) examination of the products was carried out with a Cary 60 Recording Spectropolarimeter and a Model 6001 CD attachement. Processing of the CD data was carried out by curve fitting with published sets of basis spectra, using the "MLAB" system of the PDP-10 computer. This process gives estimates of the percent of chain length existing as α-helix, β sheet, and unordered structure.
(3) Literature methods have been employed for lysozyme activity assay (1), titration of sulfhydryl groups with 5,5'-dithio-bis(2-nitrobenzoic acid) (2), "finger-printing" of proteolytic digests (3), and fluorescence quenching (4) to locate SS-containing peptides.
(4) A procedure for ion exchange chromatography, employing diethylaminoethyl (DEAE) Sephadex in 5 M urea, has been elaborated for samples of partially and fully reduced CM lysozyme.
(5) The Beckman Amino Acid Analyzer, Model 120, has been used to determine the carboxymethylcysteine content of reduced CM samples (after acid hydrolysis) as a check on the reduction level.

Findings: In a continuation of the previous work (Z01 HL 00405-06) the focus has been on ion exchange fractionation of the reduced carboxymethylated (CM) products from partial and full reduction of lysozyme, the purpose being to characterize these products with regard to content of intermediates at various reduction levels. This information is relevant to finding the stage of reduction at which lysozyme loses its native conformational characteristics and assumes those of the denatured protein.

A study of these intermediates shows that the CD behavior changes abruptly from that of the native protein to that which characterizes the fully reduced form and does so at the earliest levels of reduction studied. These results indicate a high level of cooperativity between SS bonds and secondary structure.

The finding of intermediates in the reduction of lysozyme contrasts with reports of ribonuclease (5) and other proteins (6), whereby an "all or none" effect was exhibited, for both denaturation and renaturation, meaning not only a sudden (rather than stepwise) transition between native and denatured secondary structure, but with no accumulation of SS-bonded intermediates.

The cooperativity presently indicated between SS bonds and secondary structure is consistent with the concept that most or all of the native SS bond content must form prior to a major transition to the native secondary structure.

References

1. Shugar, D. Biochim. Biophys. Acta 8, 302 (1952).
2. Habeeb, A. F. S. A., Methods in Enzymology 25B, 457 (1972).
3. Katz, A., Dreyer, W. J., and Anfinsen, C. B., J. Biol. Chem. 234, 2897 (1959).
4. Karush, F., Klinman, N. R., and Marks, R., Anal. Biochem. 9, 100 (1964).

5. Creighton, T. E., J. Mol. Biol. 129, 411 (1979).
6. Creighton, T. E., J. Mol. Biol. 137, 61 (1980).

Significance of Biomedical Research: This effort is aimed at a further elucidation of the mechanism of chain folding. Such information may contribute to understanding the relationship between primary structure and protein conformation, and make possible the reliable prediction of native conformation from knowledge of the amino acid sequence. This capability would be basic to the attack of problems relating biological functions of proteins with specific conformations of medical value. The presently reported influence of SS bonds suggests that the locations of cysteine residues within the primary structure of the reduced protein chain would be critical in the determination of secondary structure.

Proposed Course of Project: The purpose of studying SS bonds in relation to secondary structure has been concluded, and it is not anticipated that this project will be continued further.

Publications: Studies on the Relationship of Disulfide Bonds to the Formation and Maintenance of Secondary Structure in Chicken Eggwhite Lysozyme. F. H. White, Jr. Biochemistry, (accepted for publication).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO 1 HL 00409-11 LCB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Interaction of Actin and Myosin

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

PI:	Evan Eisenberg	Section Head, Cellular Physiology	LCB NHLBI
Others:	Leonard Stein	Guest Worker	LCB NHLBI
	P. Boon Chock	Research Chemist	LB NHLBI

COOPERATING UNITS (if any)

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LAB/BRANCH
Laboratory of Cell Biology

SECTION
Cellular Physiology

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 1.3	PROFESSIONAL: 1.3	OTHER: 0

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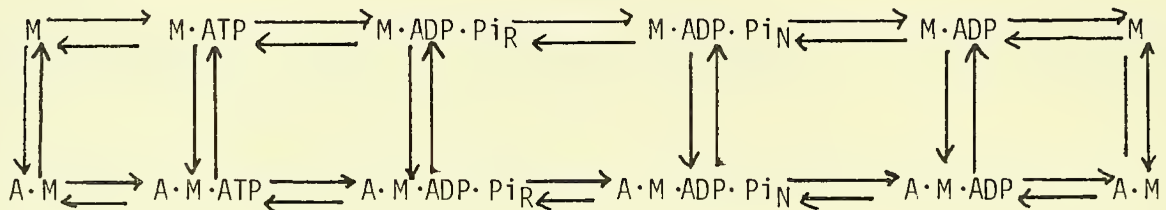
☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
Steady-state and pre-steady state kinetic studies on the interaction of actin, myosin-subfragment-one (S-1) and ATP were carried out to better elucidate the mechanism of the actin activated myosin ATPase. We have already shown that it is not required for myosin to detach from actin during each cycle of ATP hydrolysis as was originally proposed by the Lymn-Taylor model. In the present study we provide evidence for the second major feature of our kinetic model---that there is a special rate limiting step, the transition from the refractory to the non-refractory state, which preceeds Pi release and which occurs with the myosin either attached to or detached from actin. By showing that the binding constant of M·ATP and M·ADP·Pi to actin is considerably weaker than the K_{app} for ATPase activity under conditions where the magnitude of the initial Pi burst is not markedly decreased by actin, we can demonstrate that this special rate-limiting step must be present in the kinetic model. This rate-limiting step is of importance because it determines the rate at which cross-bridges enter the major force-producing state and thus it controls the velocity of muscle contraction.

PHS-6040
(Rev. 2-81)

Objectives: The accepted mechanism for the contraction of muscle *in vivo* involves the interaction of actin and myosin filaments. However, the exact nature of the energy transduction mechanism by which the energy stored in the ATP molecule (relative to ADP and Pi) is converted to useful work is not well understood. By studying the biochemical interaction of actin and myosin *in vitro* we hope to gain insight into the *in vivo* energy transduction mechanism.

Last year, we proposed a new model for the actin-activated myosin subfragment-1 (S-1) ATPase activity. This model was based on pre-steady-state and steady-state studies of the binding of S-1 to actin in the presence of ATP, and the fact that *no* significant inhibition of the actin-activated ATPase by actin has ever been observed:



where M = myosin and A = actin. In this model, instead of the S-1 molecule cycling between states bound to and dissociated from actin, the S-1 cycles between states weakly bound to actin (M·ATP, M·ADP·Pi_R and M·ADP·Pi_N) and states strongly bound to actin (M·ADP and M). As in the original refractory state model there is a special rate-limiting step (M·ADP·$Pi_R \longrightarrow$ M·ADP·Pi_N) but in contrast to the previous model, this step can occur with the S-1 either bound to or dissociated from actin.

During the past year, we have tested a key feature of this model: That there is a special rate-limiting step, the transition from the refractory to the non-refractory state, which preceeds the release of Pi and which occurs at the same rate whether the S-1 is attached to, or detached from, actin.

Methods and Findings: The major reason for postulating the existence of a special rate limiting step in our model is to explain the difference which occurs between the K_{app} for the ATPase activity (K_{ATPase}) and the binding constant of S-1 ATP and S-1·ADP·Pi to actin ($K_{binding}$). Detailed studies were carried out using both the A-1 and A-2 isoenzymes of myosin S-1. We found that, in the presence of ATP $K_{binding}$ for the A-1 S-1 was 4 times stronger than for A-2 S-1. This is the same ratio of binding constants which is found in the absence of ATP, although the binding in the presence of ATP is $\sim 10^5$ weaker than in the absence of ATP. We also found that at 15°, at very low ionic strength, K_{ATPase} is 6-8 fold stronger than $K_{binding}$ for both A-1 S-1 and A-2 S-1. There is only one way these data can be explained by a model *without* the special rate limiting transition from the refractory to the non-refractory state: that is to postulate that the hydrolysis step itself is rate limiting when S-1 is attached to actin and the subsequent Pi release step is quite fast. However, if this were the case, it would predict that the magnitude of the initial Pi burst should decrease greatly as the actin concentration is increased. To test whether this, in fact, occurs with A-1 S-1, we measured the magnitude of the initial Pi burst as a function of actin concentration, using both fluorescence measurements, and direct measurements carried out with the three syringe quench-flow apparatus. In both cases the

results show that the magnitude of the initial Pi burst was much larger than would be predicted by a model without a special rate-limiting step which preceeds Pi release. We therefore conclude that our kinetic model, shown above, is valid. We are presently preparing this work for publication.

Significance to Biochemical Research: Understanding the mechanism of the actomyosin ATPase is central to gaining an understanding of muscle contraction as well as many other motile systems. This knowledge, in turn, may have important applications in the study of muscular dystrophy and heart disease.

Proposed Course of Project: During the coming year, we plan to complete our kinetics studies which provide evidence for the special rate limiting step, the transition from the refractory to the non-refractory state. In particular, we plan to measure the magnitude of the initial Pi burst with a new three syringe quench-flow apparatus which provides better mixing than the one we are presently using. We also plan to extend our measurements of the initial Pi burst to studies with regulated actin where the steady-state ATPase rate is much slower and therefore the measurement can be made more accurately. Finally we plan to extend our studies to smooth muscle S-1 and HMM to determine if our kinetic model can account for the kinetic properties of the smooth muscle actomyosin ATPase.

Publications:

Stein, L. A., Chock, P. B., and Eisenberg, E.: Mechanism of the actomyosin ATPase: Effect of actin on the ATP hydrolysis step. Biochemistry 78, 1346-1350, 1981

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00411-06 LCB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Respiration-linked H^+ ejection by reconstituted E. coli succinoxidase vesicles.

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

PI:	Richard W. Hendler	Section Head	LCB NHLBI
Other:	Oruganti H. Setty	Visiting Fellow	LCB NHLBI
	Richard I. Shrager	Mathematician	LSMM DCRT

COOPERATING UNITS (if any)

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Laboratory of Cell Biology

SECTION
Section on Membrane Enzymology

INSTITUTE AND LOCATION
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Membrane vesicles formed from soluble components of the E. coli respiratory chain are capable of ejecting protons in response to a respiratory pulse. The H^+/O ratio in the reconstituted system is 3 to 9% that of the native membrane system. This ratio could not be increased by using a variety of different methods for reconstitution or supplementation with DCCD, succinate transport proteins or coenzyme Q_6 + menadione. It was shown that a major reason for the low measured H^+/O ratios is that the vesicles are leaky to protons and to the substrate being oxidized, succinate. The formation of a properly oriented membrane potential was shown by the dependence of proton ejection in the presence of potassium.

PHS-6040
(Rev. 2-81)

Major Findings: The previous report established that succinoxidase vesicles, reconstituted from soluble components, do achieve proper asymmetry for a net ejection of protons in response to a respiratory pulse. Experiments designed to raise the measured H^+/O ratio or to understand the reasons for its lower value than found in native vesicles continued. Additional techniques for achieving reconstitution were tried. These included injection of ethanol into the reconstitution mixture to affect reconstitution, dialysis in the presence of Sephasorb beads to form large "supported" vesicles, and the use of octyl glucoside in place of deoxycholate. None of these techniques was more effective than the earlier techniques described in the previous report. The treatment of vesicles with DCCD, which is known to "plug" proton leaks through the F_1 coupling factor, or with succinate transport proteins to enhance the uptake of the electron donor, or with coenzyme Q_6 or menadione to enhance the operation of the Co. Q cycle did not help.

Large pore-size molecular sieves were used to establish the size profile of the reconstituted vesicles and to separate a heterogenous population into fractions with possibly higher H^+/O ratios. The vesicles were entirely excluded from Sepharose 2B but were included and separated into 4 fractions by Biogel A 150 m. The H^+/O ratio was essentially the same in all peaks and electron microscopy showed they were heterogenous. Standard sized polystyrene latex beads were used to determine the sizes of the fractionated particles, but they were too strongly adsorbed by the gel column. The techniques of determining size by the trapped volume when reconstitution is done in the presence of sodium chromate was unsuccessful because of adsorption of the dye by vesicles. Columns of controlled pore glass, capable of separating large particles were tried but nore than 90% of the reconstituted vesicles were adsorbed by the glass. The same result occurred when glyceryl-coated glass was used. The average internal volume was obtained by incubating with ^3H_2O and either ^{14}C-dextran or inulin, followed by rapid centrifugation in a microfuge, rapid freezing of the pellet, and extraction of the frozen pellet with perchloric acid. The internal volume of the vesicles determined by this procedure was 0.7 μl/mg protein compared to 2.0 μl/mg protein for the spheroplasts.

It was determined that a major reason for the lower H^+/O ratio found in the reconstituted vesicles is that they are extremely leaky and cannot exclude ejected protons and/or hold an internal pool of the oxidizable substrate. This means that the degree of proper asymmetry achieved during reconstitution is higher than indicated by the measured H^+/O ratio which must be taken as a minimal value. In spite of the extreme leakiness for the vesicles for K^+ as well as for H^+, and succinate, it was demonstrated that a properly oriented membrane potential does form in response to a respiratory pulse. This was shown by the fact that vesicles could not eject protons in a medium containing a very low amount of K^+ but could when the medium was supplemented with K^+. The reason is that the inside-negative membrane potential prevents the ejection of the H^+ and only when a sufficient amount of K^+ is available to neutralize the inside-negative charges, will protons be extruded. Attempts to quantify the magnitude of ΔpH and membrane potential by the standardized techniques using flow dialysis and radioactive probes have revealed hitherto undescribed but serious artifacts in the method. We are currently exploring these problems and plan to write a paper to describe them and how to deal with them.

Proposed Course: The main objective in this project has been achieved, namely the demonstration and quantification of ejection of protons to the outside of respiratory vesicles reconstituted from soluble components. In the several months

remaining before the termination of Dr. Setty's appointment we will try to quantify the magnitude of formation of ΔpH and membrane potential in this system.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00413-05 LCB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

The Binding of the Subfragments of Myosin to Actin

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

PI:	Lois Greene	Staff Fellow	LCB NHLBI
Other:	Evan Eisenberg	Section Head, Cellular Physiology	LCB NHLBI

COOPERATING UNITS (if any)

None

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Laboratory of Cell Biology

SECTION
Cellular Physiology

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The effect of the troponin-tropomyosin complex on the binding of myosin subfragment-one (S-1) to actin was studied in the presence of different nucleotides as a test of our cooperative binding model developed in collaboration with Dr. Terrell Hill. Our model predicts that both the equilibrium constant between the weak binding form and the strong binding form of actin, and the tropomyosin interaction term should be intrinsic properties of the regulated actin filament. We found that this was the case, thereby confirming our model. In addition, we found that, in the absence of calcium, troponin- tropomyosin inhibits the binding of S-1·ADP (and S-1 alone) to actin much more than the binding of S-1·AMP-PNP. This shows that the nucleotide bound to S-1 determines how strongly troponin-tropomyosin is able to inhibit the binding of the S-1 to actin. In fact, inhibition is completely absent when the bound nucleotide is ATP. (See project no. Z01 HL 00417-02 LCB). These results also suggest that troponin-tropomyosin may inhibit the actomyosin ATPase activity by preventing the rotation of the cross-bridge from the 90° (ATP) state to the 45° (ADP) state, thereby inhibiting the release of Pi.

PHS-6040
(Rev. 2-81)

Objectives: The equilibrium binding of S-1 to the troponin-tropomyosin-actin complex (regulated actin) was examined to understand the mechanism of regulation of muscle contraction. We previously found that in the presence of ADP, S-1 binds with positive cooperativity to regulated actin, while it binds independently to unregulated actin. To describe the action of the troponin-tropomyosin complex on the binding of S-1 to actin, we developed a cooperative binding model in collaboration with Dr. Terrell Hill. In this model, the cooperative response depends on two properties of the regulated actin filament; the equilibrium constant between the weak form and the strong form of actin and the interaction term between tropomyosin molecules. As a test of our model, these properties should not change when S-1 binds to regulated actin in the presence of different nucleotides. In the present study this was examined by measuring the binding of different S-1 nucleotide complexes to regulated actin both in the presence and absence of calcium.

Methods Employed and Major Findings: The binding of S-1 to regulated actin in the presence of different nucleotides was performed by mixing (with regulated actin) varying concentrations of S-1 blocked at SH_1 with $[^{14}C]$-iodoacetamide. After centrifuging the acto-S-1 complex, the concentration of S-1 which remained in the supernatant was measured to determine the extent of binding. These studies were conducted both in the presence and absence of calcium since the calcium concentration affects the cooperative response.

Our results show that the nucleotide bound to S-1 affects the interaction of S-1 with regulated actin. In the absence of calcium, the troponin-tropomyosin complex initially causes inhibition of the binding of the S-1 and S-1·ADP to actin, but inhibits the binding of S-1·AMP-PNP to a lesser extent. However, in the presence of calcium, there is only slight inhibition in the binding of S-1 to actin, measured in the presence of either ADP or AMP-PNP. Under all conditions, when the actin beccmes saturated with S-1, the binding of S-1 to actin is greater in the presence than in the absence of troponin-tropomyosin complex. Analyzing these data using our model, we find that the cooperative response is independent of the nucleotide bound to S-1, in agreement with our cooperative binding model. We also find that the nucleotide bound to S-1 affects both the inhibition and the potentiation of S-1 binding to regulated actin. Therefore, nucleotide is able to modulate the cooperative response even though this response is an intrinsic property of the thin filament. These results are consistent with the lack of effect of the troponin-tropomyosin complex on the bindinf of S-1 to actin in the presence of ATP (See project no. Z01 HL 00417-02 LCB). It thus appears that nucleotide affects the action of the troponin-tropomyosin complex on the binding of S-1 to actin.

To determine if the effect of nucleotide was simply due to an effect on the strength of binding of S-1 to actin, binding studies were conducted comparing the effect of ATP and AMP-PNP on the binding of S-1 to regulated actin under conditions where S-1 binds with the same affinity to unregulated actin in the presence of these two nucleotides. This was achieved by working at different ionic strengths: μ=0.18 M in the presence of AMP-PNP and μ=0.018 M in the presence of ATP. We found that there was cooperativity in the binding of S-1 to regulated actin in the presence of AMP-PNP at μ=0.18 M, while there was no evidence of cooperativity in the binding in the presence of ATP at μ=0.018 M. The difference in ionic strength is not causing this difference in the action of the troponin-tropomyosin since even at a very low ionic strength, there was cooperativity in the binding of S-1·AMP-PNP to regulated actin. Therefore, these results suggest that S-1 binds to actin in

different conformations, perhaps different angles, in the presence of ATP and AMP-PNP since the action of the troponin-tropomyosin complex on the binding of S-1 to actin differs so greatly with these two nucleotides. From these findings, we have developed the following mechanism for muscle relaxtion: the troponin-tropomyosin complex causes relaxation not by blocking the binding of S-1·ATP or S-1·ADP·Pi to actin as suggested by the steric blocking model, but rather by inhibiting the release of Pi from the acto·S-1·ADP·Pi complex. Since the troponin-tropomyosin complex profoundly weakens the binding of S-1·ADP, the inhibition of Pi release may be caused by the cross-bridge being hindered in its rotation from the 90° state (A·M·T or A·M·D·Pi) to the 45° state (A·M·D). Therefore, the troponin-tropomyosin complex may be preventing rotation of the cross-bridge in muscle, thereby inhibiting the Pi release.

The equililbrium binding of S-1 to actin has also been examined in the presence of pure tropomyosin. The cooperative binding observed was not significantly different from that obtained with the troponin-tropomyosin complex in the presence of calcium. Therefore, troponin may only affect the cooperative binding of S-1 in the absence of calcium; in the presence of calcium it appears to have no effect.

In another study, the binding of myosin and heavy meromyosin (the two-headed soluble fragment of myosin) to actin were compared under identical conditions to determine whether the tail of myosin affects its ability to bind actin. This experiment was conducted under high ionic strength conditions where myosin exists predominantly as monomer. The binding of HMM to F-actin was very similar to that obtained with myosin both in the presence and absence of ADP. In addition, in the presence of the troponin-tropomyosin complex, both HMM and myosin show the same cooperative binding to regulated actin when examined in the absence of calcium and the presence of ADP. This shows that the myosin tail does not affect the binding of the myosin head to actin.

Significance: The interaction of actin with myosin in the presence and absence of the regulatory proteins provides information on the basic mechanism of muscle contraction. An understanding of the regulation of contraction is critical in the study of diseases of skeletal, cardiac, and vascular muscle.

Proposed Course of Research: The plan for this project is to continue examining the binding of S-1 to regulated actin to determine whether our model is valid for the equilibrium binding of S-1 to regulated actin. Pre-steady state experiments will examine the kinetics of S-1 binding to regulated actin. These experiments will be conducted under the same conditions as we used in our equilibrium binding studies. We will measure the rate of S-1 binding to actin at varying S-1 and actin concentrations. These rates will be correlated with our equilibrium data to determine whether they fit our model.

Another aspect of this project is to determine the relationship between the weak form of actin and the ATPase activity of regulated acto-S-1, i.e., to determine whether the amount of weak form present, either in the presence or absence of calcium, is sufficient to account for the inhibition of the ATPase activity. In collaboration with Dr. Joseph Chalovich the proportion of actin in the weak form in calcium will be correlated with the actin activation of regulated acto-S-1 in the presence of calcium. In addition, ATPase activities of acto-S-1 and binding studies will be conducted in the presence of tropomyosin using *Acanthamoeba* actin. Since there is no inhibition of ATPase activity with *Acanthamoeba* actin in the presence of tropomyosin, it is important to establish whether S-1 binds cooperatively to the tropomyosin-*Acanthamoeba* actin complex.

In addition, the binding of HMM to actin will be studied under conditions where the second head of HMM does not bind to actin (high ionic strength and the presence of AMP-PNP). By examining the mobility of the heads of HMM, we will determine whether under these conditions, the second head becomes immobilized. The binding of myosin to actin will also be examined at low ionic strength to determine the role of filament formation on the binding of the myosin head to actin.

Publications:

Greene, L. E.: Comparison of the binding of heavy meromyosin and myosin subfragment-one to F-actin. Biochemistry 20, 2120-2126, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00415-02 LCB

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Models of Muscle Contraction

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

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.60	.60	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Modelling of several aspects of muscle contraction is presently underway. First, we are quantitatively modelling our new cross-bridge model which is based on the concept that the myosin cross-bridge oscillates between states weakly bound to actin with a preferred angle of about 90° and states strongly bound to actin with a preferred angle of about 45°. The former states have bound ATP or ADP + Pi while the latter states have no bound Pi. The concept of two major types of cross-bridge states is combined with the biochemical concept of rapid equilibria between attached and detached states and the physiological concept of an elastic cross-bridge to allow a model in which the myosin does not detach from actin during each cycle of cross-bridge action but does mechanically detach when the muscle is shortening. In addition to modelling cross-bridge action in vivo, we are currently modelling the cooperative actions of the troponin-tropmyosin actin complex, in particular the cooperative binding of myosin ADP and the cooperative effect of troponin-tropomyosin on the steady-state actin-activated myosin ATPase activity. Finally, we are comparing our current models of muscle contraction with other energy transduction systems such as ion transport to determine similarities and differences between the various systems.

Project Description

Objectives: It is now generally recognized that contraction of muscle involves the interaction of the two proteins, actin and myosin, with ATP. Based on our experimental work, both with ATP and with ATP analogues, we have developed a kinetic model for the actomyosin ATPase which has several distinguishing features. First, the binding of ATP does not irreversibly dissociate the actomyosin complex during each cycle of ATP hydrolysis; myosin-ATP is in rapid equilibrium with actomyosin-ATP. Second, ATP hydrolysis can occur without dissociation of the actomyosin complex. In fact, the ATP hydrolysis step may be even faster when the myosin is attached to actin than when it is dissociated from actin. Third, the slowest step in the cycle is the transition from the refractory to the non-refractory state which precedes the rapid release of Pi and can occur with the myosin either bound to or dissociated from actin. The theoretical formalism of T. L. Hill provides a method of relating this kinetic model to cross-bridge behavior in vivo. We have previously published a qualitative model of cross-bridge action based on this kinetic model and are presently developing a quantitative version of this in vivo cross-bridge model.

We are also modelling the cooperative action of the troponin-tropomyosin complex in vitro. This modelling is directly related to our experimental observations on the cooperative binding of S-1, and its various nucleotide complexes, to regulated actin filaments.

Methods Employed and Major Findings: The cross-bridge model which we are presently quantitatively modelling has the following major features. First, and most important, the myosin cross-bridge does not undergo a simple attachment-detachment cycle in vivo. Rather, the cross-bridge cycles between states weakly attached to actin at about 90° and states strongly attached to actin at about 45°. Since there is no mandatory detachment step, the cross-bridge only detaches when it is elastically distorted and is thus in rapid equilibrium with an attached state. Second, the rapid release of Pi is associated with the transition from the 90° to the 45° state and the rapid recovery of force in the isomeric transient. Third, the rate-limiting transition from the refractory to the non-refractory state controls the shortening velocity by limiting the number of bridges which make the transition from the 90° to the 45° state and thus go through a complete force-producing cycle.

One of the most important features of our quantitative modelling is that, for the first time, we are including almost all of the biochemical states observed in vitro. Therefore, in this new model there is a quantitative correspondence between the relative equilibrium levels of biochemical states in vitro and their relative free energy in vivo. At the present time we have quantitatively modelled both the rapid isometric transient and the steady-state force-velocity curve. Only a few details remain (in particular the rate of the isometric transient as a function of the amount of release) before this work will be ready for publication.

Since we are now reasonably certain that our kinetic model for the actomyosin ATPase is valid, we have been interested in comparing this kinetic model and the simple cross-bridge model based on it, with other ATP utiliyzing energy transduction systems. In particular, we have examined the cyclic properties of ion transport models and found that, in certain key aspects, they are similar to our muscle model. The most important feature of all of the energy transduction systems is that there is no particular step in the kinetic cycle where energy transduction takes place. Rather, the production of ADP and Pi from ATP is coupled to a process

in which the enzyme passes through a series of states or conformated changes in an ordered fashion, and it is this process which causes energy transduction to take place, rather than any particular step. We have presented this concept in a review which is now in press in Quarterly Reviews of Biophysics.

Turning to our modelling of the cooperative effects of troponin-tropomyosin, we have published a simple model in which each tropomyosin-troponin unit (including seven sites of the actin filament) can be in one of two possible states, which have different intrinsic free energies and different binding constants for S-1.

The key feature of this model is that the tropomyosin molecule can only occur in two positions on the F-actin filament. Intermediate positions are not allowed but there is always an equilibrium between the two allowed positions. We have found that this simple model can account for the cooperative binding data which we observe with S-1 and S-1 AMP-PNP as well as with S-1 ADP. However, we were interested in determining whether a somewhat different but equally simple model could also explain the data; a model in which the tropomyosin can exist in a continuum of positions on actin but under any particular condition i.e. with a particular S-1 nucleotide complex bound, the tropomyosin can occur in only one position not in an equilibrium between two positions as in the previous model. We find that this alternative model, which is presently being prepared for publication, can also account for our data. However, we have also found that certain predicitons of the two models differ and therefore we are presently designing experiments which will allow us to distinguish between the two models.

In addition to this theoretical work on the cooperative equilibrium binding of S-1 to regulated actin, as influenced by Ca^{2+}, we have extended our modelling efforts to the cooperative steady-state ATPase activity of myosin subfragment-1 on regulated actin. Exact solution of the general steady-state problem requires Monte Carlo calculations which we are beginning to perform. However, using approximations, we have examined three interrelated special cases and in a paper in press in Biophys. J. sample computer (not Monte Carlo) solutions are given. The eventual objective of this modelling effort is to apply our model to *in vitro* experimental data and to *in vivo* muscle models.

Proposed Course of Project: We will complete the quantitative modelling of our new cross-bridge model and write it up for publication. In addition, we will continue our modelling of the cooperative actions of the troponin-tropomyosin complex. In particular we will attempt to develop a model for relaxation *in vivo*. Finally, we plan to write a simple review which will present our current model of muscle contraction and relate it to other ATPases and energy transducing systems.

Publications:

Hill, T. L., Eisenberg, E., and Chalovich, J. M.: Theoretical models for cooperative steady-state ATPase activity of myosin subfragment-1 on regulated actin. Biophys. J. (in press)

Hill, T. L., and Eisenberg, E.: Can free energy transduction be localized at some crucial part of the enzymatic cycle? Quart. Rev. Biophys. (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00416-02 LCB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Studies on Plasma Proteins in Cystic Fibrosis (CF)

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

PI: Frederick H. White, Jr. — Research Chemist — LCB NHLBI

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.8	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

As an approach to elucidating the genetic defect in cystic fibrosis (CF), an investigation has been continued on reported differences in plasma $\alpha_2$macroglobulin (α_2M) from CF patients and normal individuals. The reportedly lower trypsin inhibitory acitivity in CF α_2M could not be confirmed. However, a reported lower digestibility of CFα_2M by trypsin, is in evidence. Also confirmed is a lower sialic acid content in CF α_2M. On the other hand, it is found that enzymatic removal of the sialic acid content from normal α_2M does not result in the observed differences in its interaction with trypsin, and hence factors other than the amount of incorporated sialic acid are responsible for the observed differences. One such factor may be the locations of the sialic acid residues within the protein, as is suggested by differences in the extent of removal of sialic acid with neuraminidase digestion. Thus, the reaction is approximately 90% complete for the normal protein but only 60-70% complete for the CF protein. Work continues on the investigation of sialic acid content as a possible source of observed differences between normal and CF α_2M.

PHS-6040
(Rev. 2-81)

Objective: To isolate and examine the plasma proteins, from normal and cystic fibrosis (CF) patients, in a search for structural differences related to the genetic defect in CF.

Methods:

(1) Established methods (1,2,6) have been tried in the purification of α_2M from human plasma.
(2) Literature methods have been employed for the assay of α_2M for content of sialic acid (4,10,12), hexose (11), and fucose (3,9).
(3) Immunochemical reaction of α_2M with anti-α_2M antibody has been tried to verify isolation of α_2M by the various methods of purification employed (8).
(4) The method of Lowry (5) has been used as a means of determination of protein concentration for the purpose of checking extinction coefficients.
(5) The methods of Ashwell (4) have been employed in the enzymatic removal of sialic acid from α_2M.
(6) Assays for trypsin activity after inhibition with α_2M were conducted with benzoyl arginyl ethyl ester (1) and benzoyl arginyl p-nitroanilide (7) as substrates.
(7) Gel filtration chromatography (1) was employed in the separation of products from trypsin digestion of α_2M, as a means of following the extent of digestion.

Major Findings:

(1) Investigation of published work. This study was necessary to resolve conflicting literature reports from comparative studies on α_2M from normal and CF individuals.

a. Three methods of preparing α_2M have been investigated. The methods of Shapira *et al.* (1), and Ben Yosef *et al.* (2) have failed to yield purified protein with a consistently positive reaction to anti-α_2M antibody. The method of Willingham *et al.* (6), however, has proven consistent in this respect and has been used for further study.

b. The observations of Shapira *et al.* (1), that α_2M from CF patients has lower proteolytic inhibitor activity has been investigated further, but no such differences could be confirmed.

c. These authors also report a lower digestibility of CF α_2M with trypsin as compared to the normal protein. This finding is confirmed in the present effort.

d. Reports that sialic acid content in α_2M is lower in CF (1,2) are confirmed by the present work, with 40-60% of the normal sialic content appearing in the CF protein.

(2) The possibility has therefore been considered that the lower sialic acid content may be responsible for the observed differences in α_2M (lowered digestibility with trypsin). Thus, the sialic acid content has been removed by digestion with neuraminidase. The product, however, was no different from normal samples, except for a slightly increased susceptibility to proteolytic digestion. Therefore, factors other than the amount of sialic acid contained must be responsible for the observed differences between CF and normal α_2M.

(3) The following observation is relevant to the posssiblity that the locations of sialic acid residues within the protein could be such a factor. Differences

in digestibility of the sialic acid content with neuraminidase, as compared to acid digestion, indicate for the normal protein that the sialic acid content is approximately 90% removed from the former, whereas the latter treatment results in virtually complete removal. For the CF protein, however, the removal of sialic acid with neuraminidase has been only 60-70% complete. This difference suggests that the locations of the sialic acid residues may be different, resulting in a decreased susceptibility to removal by enzymatic digestion.

(4) Assays for other carbohydrates in α_2M (fucose and hexose) do not indicate significant differences between normal and CF proteins.

References:

1. Shapira, E., Martin, C. L., and Nadler, H. L. (1977) J. Biol. Chem. 252, 7923.
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6. Willingham, et al. (1980) J. Histochem. Cytochem. 28, 818.
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12. Warren, L. (1959) J. Biol. Chem. 234, 1971; Nature 186, (1960) 237.

Significance to Biomedical Research: The genetic defect of CF remains unknown, and its elucidation could be significant in producing a more effective treatment, as well as diagnosis, of this disorder. The present effort is aimed at understanding this defect.

Proposed Course: We shall investigate further the differences in normal and CF α_2M, with emphasis on the carbohydrate content, and may extend the study to include other plasma proteins.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00417-02 LCB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

The Mechanism of Regulation of Acto-S-1 ATPase by Troponin-Tropomyosin

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

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Others:	Joseph Chalovich	Staff Fellow	LCB NHLBI
	P. Boon Chock	Research Chemist	LB NHLBI

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Laboratory of Cell Biology

SECTION
Cellular Physiology

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.90	.90	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The widely accepted steric blocking model of vertebrate skeletal muslce relaxation suggested that myosin subfragment-one (S-1) does not bind to regulated actin (actin + troponin + tropomyosin) in the presence of ATP under relaxing conditions (low Ca^{2+}) where ATP hydrolysis is inhibited. We have shown previously by stopped-flow measurements and now by direct binding studies that S-1 binds to regulated actin with about the same affinity in both relaxing and activating conditions. At pH 7.0, 25° and 18 mM ionic strength in activating conditions the rate of ATP hydrolysis is 20 times greater than in relaxing conditions although the association constant of S-1 to regulated actin in the presence of ATP, is about 1.5×10^4 M^{-1} in both cases. We have observed similar behavior at higher (50 mM) ionic strength. Also, we have shown that in relaxing conditions the rate of ATP hydrolysis by S-1 is inhibited because of a large decrease in the V_{max}. These data do not support the steric blocking model of muscle relaxation, rather they suggest that in relaxing conditions, the regulatory proteins troponin and tropomyosin inhibit a rate process in the cycle of ATP hydrolysis.

PHS-6040
(Rev. 2-81)

Project No. Z01 HL 00417-02 LCB

Project Description

Objectives: To confirm our earlier observation that regulation of muscle contraction by troponin-tropomyosin does not occur by altering the binding constant of S-1 to actin in the presence of ATP.

Methods Employed and Major Findings: Binding of S-1 to actin or regulated actin, in the presence of ATP, was measured by rapidly sedimenting the acto·S-1 complex in an air-driven ultracentrifuge. The amount of unbound S-1 was determined by measuring the ATPase activity in the supernatant. Binding constants were determined by measuring the fraction of S-1 bound over a wide range of actin concentrations. Under identical conditions the steady state rate of ATP hydrolysis by regulated acto·S-1 was measured by the rate of liberation of [^{32}P] Pi from [γ-^{32}P] ATP or by the rate of proton liberation using a pH stat. At pH 7.0, 25° and μ = 18 mM in the presence of Ca^{2+} (activating conditions) the rate of ATP hydrolysis is >20 times larger than in the absence of Ca^{2+} (relaxing conditions). In contrast, the association constant of S-1·ATP and S-1·ADP·Pi with regulated actin is virtually the same in the absence of Ca^{2+} (1.4×10^4 M^{-1}) as in the presence of Ca^{2+} (1.5×10^4 M^{-1}). A similar study was carried out at a more nearly physiological ionic strength of 50 mM. Under this condition the ATPase rate is inhibited 98% in the absence of Ca^{2+} although the association constant is not significantly different from that measured in the presence of Ca^{2+}. Steady state ATPase rates have been measured in relaxing conditions as a function of actin concentrations at various levels of saturation of the actin filament with troponin-tropomyosin. In the absence of troponin-tropomyosin or in the presence of Ca^{2+} the ATPase activity is high but at saturating levels of troponin-tropomyosin the rate is low. The progressive level of inhibition is accompanied by a large decrease in the maximum velocity of the reaction but only a small change in the apparent binding constant. This supports the binding studies in that troponin-tropomyosin regulates contraction by modulating a rate process and not by altering the assocation constant of S-1 to actin.

Preliminary studies have been done to determine which step in the pathway of ATP hydrolysis is modulated by troponin-tropomyosin. Using stopped-flow fluorescence and a three-syringe rapid quenching device we have early indications that troponin-tropomyosin has no effect on the initial Pi burst.

Studies on the effect of tropomyosin (in the absence of troponin) on the actin activated S-1 ATPase rate have been initiated. Preliminary results show that the mild inhibition that is caused by tropomyosin alone is primarily due to a decrease in the maximum velocity of the reaction. This supports our more detailed study with the troponin-tropomyosin complex.

Significance to Biomedical Research: Knowing the mechanism of regulation of skeletal muscle contraction is important in determining the etiology of various muscular dystrophies and may have significance in the future for the treatment of these disorders. This work also has applicability to the study of heart and vascular disease since it now appears that certain aspects of regulation (i.e. modulation of a rate process) may be shared by other muscle types such as smooth muscle.

Proposed Course of Research: (1) A more detailed study of the effect of tropomyosin and troponin-tropomyosin on the burst rate of ATP hydrolysis will be done in an attempt to localize the kinetic step which is modulated by these

regulatory proteins. (2) The effect of pure tropomyosin on the acto-S-1 ATPase rate will be studied in greater detail. (3) The regulated actin filament exists in two states, one characterized by a low ATPase rate and another state characterized by a higher ATPase rate. An attempt will be made to shift the actin filament completely to one state or the other so that the kinetics of ATP hydrolysis of both states can be studied independently. (4) S-1·ADP (S-1 with bound ADP) binds to the actin filament in what may be imagined as a 45° angle and it appears that the actin filament can be completely saturated with S-1·ADP. There is evidence that S-1·ATP and S-1·ADP·Pi associates with actin in a different manner than S-1·ADP; the angle of attachment may be ~90° and the bound S-1 may be less rigid and less ordered. Studies will be initiated to determine whether the binding stoichiometry of S-1·ATP and S-1·ADP·Pi to actin is one S-1 per actin monomer as it is with S-1 and S-1·ADP.

Publications

Chalovich, J. M., Chock, P. B., and Eisenberg E.: Mechanism of Actin of Troponin-Tropomyosin. J. Biol. Chem. 256, 575-578, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00418-01 LCB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Electrochemical potentials of protons in energy-transducing membranes.

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

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	Robert F. Bonner	Physicist	BEIB DRS
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TOTAL MANYEARS: 0.8	PROFESSIONAL: 0.8	OTHER:

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A program has been started to develop equipment and methods for monitoring instantly the membrane potential and pH differences across a respiring membrane. In addition, other important quantities such as the numbers and ratios for movements of ionic species across the membrane will be monitored. Thus far, we have constructed an electrode specific for the membrane potential sensitive probe, TPP^+, and developed computer programs which show that membrane potential can be instantly followed by electrochemical means.

PHS-6040
(Rev. 2-81)

Background: Energy released during respiration is converted into an electrochemical potential for protons ($\Delta\bar{\mu}_{H}+$), which is also referred to as a proton-motive force or PMF. The $\Delta\bar{\mu}_{H}+$ consists of two components, a membrane potential, $\Delta\Psi$, and a pH difference maintained across the membrane, ΔpH. The measurement of these components is cumbersome and time consuming. The most widely used technique employs flow dialysis and radioactive probes which distribute themselves between the intra- and inter-vesicular spaces according to $\Delta\Psi$ and ΔpH. The determinations require the use of a fraction collector and the taking and counting of many fractions. Results are known many hours after completion of the experiment. Our new approach for the measurement of $\Delta\bar{\mu}_{H}+$ uses selective electrodes and a microcomputer to instantly convert electrode readings into $\Delta\psi$ and ΔpH. In this way, the investigator can follow changes in these parameters in response to additions to the reaction mixtures during the course of an experiment.

Major Findings: The small organic cation, tetraphenyl phosphonium (TPP^+) has been widely used in flow dialysis as a probe for $\Delta\Psi$. The literature describes the construction of an electrode which responds to TPP^+. We have constructed such an electrode and made significant improvements in its design. The electrode responds in a Nernstian manner to TPP^+ concentration. We have also purchased and tested an electrode which is highly selective for K^+ which is a different probe for $\Delta\Psi$. The computer systems laboratory had provided us with a single board microcomputer (SBC) and a highly sophisticated mathematical operations board. They have also constructed suitable analog to digital (A/D) and digital to analog (D/A) interfaces. We have developed our own software which enables us to link the SBC to our existing microcomputer which has full software development capabilities and which in turn is linked to the NIH computer system. We have also written programs which operate the A/D and D/A interfaces and the mathematics unit. The mathematics unit converts the measured voltage changes, ΔE, and predetermined internal and external vesicular volumes v and V, into $\Delta\Psi$ according to the relationship,

$$\Delta\Psi = -59 \log \left(\frac{(V + v)}{V\ 10^{(\Delta E/59)}} - 1 \right) \quad -59 \log \frac{V}{v}$$

The $\Delta\Psi$ quantity in mVolts is then sent to a recorder. An experiment using *E. coli* membrane vesicles was performed in duplicate. One was assayed by the standard procedure using flow dialysis and the counting of radioactive samples whereas the other used the electrode, computer system. Both systems showed the same response of $\Delta\Psi$ to manipulations in the experiments. Therefore, except for some additional refinements, the goal of instantly monitoring the $\Delta\Psi$ has been achieved.

The measurement of ΔpH is much more difficult because of the buffering capacities of the inner and outer compartments. Flow dialysis uses weak organic acids which distribute across a membrane vesicle according to the ΔpH. We have tried unsuccessfully to develop electrodes specific for the organic anions. An alternate approach is to use a pH electrode and to determine the buffering capacities as a function of pH for both the inner and outer vesicle compartments. These measurements are based on the fact that added acid or base is quickly buffered by the external surface of the vesicles and medium and only more slowly

by the internal medium, and by the effects of proton ionophores in these titrations.

Proposed course: Dr. Robertson who arrived in this laboratory in November, 1980, is leaving about June 15, 1981. Therefore, the proposed course is somewhat uncertain. Specific goals are the successful construction of electrodes specific for organic acid anions and/or the determination of intra- and inter-vesicular buffering capacities. Once either or both of these goals are achieved, suitable software will be developed for the monitoring of ΔpH. Other goals include the use of the input from an oxygen uptake and the ratios of movements of specific cations and protons to each other and to the uptake of oxygen.

Publications: none

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00419-01 LCB

PERIOD COVERED
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TITLE OF PROJECT (80 characters or less)

Structure-function relationships in eucaryotic cells

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

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Rowena Roberts

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TOTAL MANYEARS: 2.5	PROFESSIONAL: 1	OTHER: 1.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Acanthamoeba ingests both digestible particles (yeast) and indigestible particles (latex beads) readily. Experiments to test whether internal processing of digestible and indigestible particles is also similar showed that the cell has mechanisms for distinguishing particle quality and with the appropriate stimulus can rid itself more rapidly of indigestible particles. These experiments are part of a continuing study of cellular controls on endocytosis.

In studies on another eucaryote, Saccharomyces cerevisiae, long term objectives are to understand the spatial and temporal control of chitin synthase activity during the cell cycle. An examination of seven temperature sensitive mutants blocked in various stages of the cell cycle has shown that mutants blocked in early stages of the cell cycle synthesize abnormally high amounts of chitin and show a loss of spatial restriction of the sites of synthesis.

PHS-6040
(Rev. 2-81)

Objectives: We have initiated studies which attempt to approach the problem of how Acanthamoeba controls endocytic rates, and thus the rate of membrane recycling.

In unrelated studies on the yeast Saccharomyces cerevisiae, we are studying control and localization of chitin synthase activity during the cell cycle.

Methods Employed: Transmission electron microscopy is being used for morphological studies of fixed and embedded cells and for examination of purified proteins by negative staining. Phase and fluorescence light microscopy are used to monitor living cells. Standard biochemical procedures are used for enzyme activity and protein measurements. Cytochemical staining of thin sections was performed using colloidal gold stabilized with appropriate ligands.

Major Findings:

1. We have begun experiments to answer whether endocytic rates depend primarily on internal factors or whether they are related to surface stimuli (e.g. binding of particles or other ligands to the surface). Experimentally we deal with three conditions of endocytosis: pinocytosis, or fluid uptake; phagocytosis of indigestible particles (latex beads); and phagocytosis of digestible particles (yeast). Since Acanthamoeba bind and ingest yeast and latex beads equally well, it is appropriate to ask if cells "recognize" and handle non-digestible particles differently than digestible particles once they are internalized. Two types of experiments address this question. Cells were pre-loaded with yeast, washed free of excess yeast, reincubated with latex beads, and monitored for bead uptake. Some digestion of yeast took place during the experiment, but there was no significant decrease in the number of yeast per cell. Such loaded cells did not take up beads, whereas control cells took in beads at a rapid rate. In the converse experiment, cells were loaded with beads, washed and reincubated with yeast. In the pre-loaded cells yeast uptake was comparable to controls and a rapid loss of beads was observed concomitant with yeast uptake. On the other hand cells pre-loaded with beads but not exposed to yeast did not exhibit a similar rapid rate of bead loss.

These experiments suggest that the cell does in some way distinguish between digestible and non-digestible particles, and that surface stimuli to ingest (i.e. particle binding) may be over-ridden by internal signals from digestible particles, but not by indigestible particles. These initial findings indicate that this type of experiment can be informative.

2. Last year we reported the successful use of colloidal gold stabilized with wheat germ agglutinin (WGA) to localize chitin in thin sections of conventionally fixed, Epon embedded yeast cell walls. We have used this technique to localize chitin in normal cells and show at high resolution that the localization is that predicted by methods of much lower spatial resolution, namely combinations of selective extractions, chemical analysis and morphological examination of the residues. To learn more about the timing of initiation and suppression of chitin, synthase activity during the cell cycle, we have examined seven temperature sensitive mutants that are blocked at various stages of the cell cycle. The amount of chitin in the cell wall of each mutant was measured (R. Roberts) and the localization of that chitin in the wall was determined electron microscopically by colloidal gold-WGA staining of thin sections. Five mutants blocked early in the cell cycle showed overproduction of chitin and abnormal localization of chitin (over the entire wall). At permissive temperatures each of these mutants showed the wild-type distribution of chitin, i.e. restriction of chitin to the bud scar region. Interpretation of these findings in terms of operative controls over chitin synthase requires further experiments.

3. Assistance has been provided to other members of the laboratory by examining purified proteins and monitoring membrane isolations by electron microscopy. With Altaf Lal we have followed progress in isolation of membranes and cell ghosts. With J. H. Collins we have examined the effect of Mg concentration on filament size in purified Acanthamoeba myosin II, determined the capacity of tryptic fragments of Myosin II to form filaments, and looked at the effects of phosphorylation on filament formation. We have also examined protein samples by electron microscopy for S. Mockrin, J. Albanesi and O. H. Setty.

Proposed course of the projects: We will continue to investigate cellular mechanisms that control endocytic uptake and membrane recycling. Experiments are planned to look at chitin synthesis on isolated membranes to determine whether vectorial transport of the completed polymer is maintained.

Publications:

Bowers, B., T. Olszewski, and J. Hyde. Morphometric analysis of volumes and surface areas in membrane compartments during endocytosis in *Acanthamoeba*. J. Cell Biol. 88: 509-515. 1981.

Bailey, C. F. and B. Bowers. Localization of lipophosphonoglycan in membranes of Acanthamoeba by using specific antibodies. Mol. Cell Biol. 1: 358-369. 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00501-07 LCB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Actin of Nonmuscle 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)

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LAB/BRANCH
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SECTION
Section on Cellular Biochemistry and Ultrastructure

INSTITUTE AND LOCATION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.5	4.5	0

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

By analysis of the kinetics of its stimulation by cytochalasins, it has been established that monomeric actin catalyzes the hydrolysis of ATP. This necessitates the existence of an actin·ADP·Pi species as an intermediate that will be present under polymerizing conditions and might be involved in the polymerization reaction. Covalently crosslinked actin dimer has been prepared and analyzed. Its spectral properties suggest it may be in the conformation of F-actin. The dimer binds two moles of nucleotide, ATP more strongly than ADP, and they exchange at rates intermediate between the exchange rates of G-actin and F-actin. One site exchanges more rapidly than the other; this site contains about 15% bound ADP·Pi and 85% bound ATP. It is possible that the dimer is a model of the ends of actin filaments. Profilin has been shown to inhibit the ATPase activity of monomeric actin as well as the ATPase activity that accompanies actin polymerization. The K_D for the 1:1 complex of actin and profilin has been found to be 5-10 X 10^{-6}M. Profilin inhibits elongation as well as nucleation and can depolymerize F-actin as well as inhibit the polymerization of G-actin.

PHS-6040
(Rev. 2-81)

Project Description

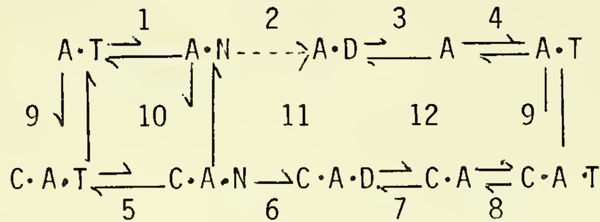
Objectives: Actin is the major protein in the cytoskeleton of all eukaroytic cells. It is essential for many different motile processes and for the regulation of cell shape and maintenance of cell organization. Actin is a globular protein of molecular weight 42,000 that polymerizes into double-stranded helical filaments under physiological conditions. Filaments are the functional form of actin. In non-muscle cells, the equilibrium between monomeric and polymeric states of actin is regulated through the interaction of actin with several other proteins, one of which is profilin. When actin polymerizes, one molecule of ATP is hydrolyzed for every molecule of actin that is converted from monomer to polymer. The energy of ATP is not utilized in the polymerization process, however, and actin with an associated non-hydrolyzable nucleotide can polymerize essentially as well as actin with bound ATP. Therefore, it becomes important to discover the role of ATP hydrolysis in the polymerization of actin. One possibility derives from the fact that irreversible hydrolysis of ATP at both ends of the actin filament makes the polymerization a steady state process rather than an equilibrium process. At steady state, net addition of actin protomers can occur at one end of the filament while equivalent net loss of actin protomers occurs at the other end of the filament. The system as a whole is at equilibrium but no reaction at either end of the filament is at equilibrium. As a consequence, on average an actin protomer will add to one end of the filament and eventually be lost from the other end. Several possible roles for this filament translocation have been proposed. If, as seems likely to be true, however, one or both ends of the filaments are blocked in situ by "capping" proteins, actin filament translocation could not occur. An alternate possibility is that the role of ATP hydrolysis is to allow for regulation of the polymerization process through kinetic modulation of any of the several steps in the ATPase reaction.

Methods employed and major findings: (1) Monomeric actin ATPase cycle: Theoretical considerations require that there be an ATPase cycle catalyzed by monomer alone and by polymer ends alone and that the accelerated ATPase rate when monomer and polymer interact be due to the combination of the complementary faster portions of each separate cycle. We had previously discovered that the low ATPase activity of monomeric actin was greatly stimulated by cytochalasins, drugs that inhibit actin polymerization primarily (it is believed) by binding with high affinity to, and blocking, one end of the actin filament. We have now confirmed that the cytochalasin-stimulated actin ATPase activitiy is due to monomeric actin alone by demonstrating, both for muscle and amoeba actin, that the cytochalasin-stimulated ATPase activity is directly proportional to the concentration of monomeric actin both below the critical actin concentration (where no actin filaments are present) and above the critical concentration (where monomer concentration remains constant with all additional actin polymerizing). In contrast, the ATPase activity of actin in the absence of cytochalasin is proportional to the actin concentration below the critical concentration but continues to increase above the critical concentration (when the concentration of actin monomer does not increase) because of the ATPase activity associated with the continued interaction of actin monomers with filament ends. This monomer-polymer interaction is blocked by cytochalasins.

We then carried out a detailed kinetic analysis of the ATPase activity of monomeric actin as a function of the concentration of four different cytochalasins (B, C, D, and E). In each case, the reciprocal plot of ATPase activity versus cytochalasin concentration was linear, showing that essentially all of the cytochalasin was free in solution as expected from the inability to detect binding

of cytochalasin to actin monomers. Interestingly, the V_{max} values, obtained by extrapolating the ATPase activities to infinite concentrations of the cytochalasins, were essentially the same for all four cytochalasins but the Kapp values (the concentrations of cytochalasins required to reach half-maximal ATPase activities) varied by as much as 400-fold. When the cytochalasin concentrations were raised to very high levels (much greater than Kapp), the reciprocal plots were no longer linear. For some of the cytochalasins, the observed ATPase rates were greater than the extrapolated Vmax values while for other cytochalasins the ATPase activities were inhibited.

The simplest model we could derive to explain these data is as follows, where A=actin, T=ATP, D=ADP, N=nucleotide, and C=cytochalasin.

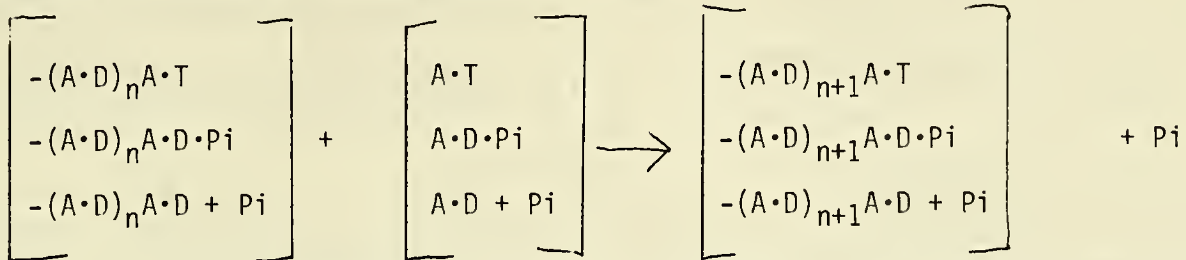


According to this scheme, the first step of the monomeric actin ATPase cycle in the absence of cytochalasin would be the hydrolysis of actin-bound ATP to actin-bound products actin·ADP·Pi, represented in the scheme as A·N because the step may also include either or both of the conformational intermediates A*·T or A*·D·Pi, preceding or following the formation of A·D·Pi. Following the hydrolysis step (step 1), there would be a very slow, rate-limiting release of bound Pi (step 2), then the faster loss of bound ADP (step 3) and, finally, the rapid re-binding of ATP (step 4). The cytochalasins would accelerate the reaction by binding to the A·N species (step 10) so that the faster steps 10, 6 and 11 would replace step 2. According to the scheme, at Vmax all of the A·N would be in the form of C·A·N and the rate limiting step would now become step 1 which is independent of cytochalasin. This would explain why the same Vmax value was obtained for all of the cytochalasins tested. The complete ATPase cycle would now be steps 1, 10, 6, 11, 3 and 4. Because the equilibrium at step 1 apparently lies very far to the left, it has not been possible to demonstrate directly the binding of cytochalsins to monomeric actin. The association constants of the cytochalasins for A·N could be very different so that they could have very different Kapp values despite the near identity of the Vmax values. Eventually, when the concentrations of the cytochalasins are raised to very high values, all of the actin would be bound to cytochalasins and the ATPase cycle would be steps 5, 6, 7, and 8. The observed ATPase rate would be greater or lesser than the extrapolated Vmax value depending on whether step 5, for the particular cytochalasin, were faster of slower than step 1. Binding of cytochalasin to monomeric actin would still go undetected because the bound cytochalasin would be too small a fraction of the total cytochalasin to detect.

As a consequence of these results, it can now be stated that, under conditions of actin polymerization, the species actin·ADP·Pi must be present in addition to actin·ATP and actin·ADP. In addition, either or both an actin*·ATP and actin*·ADP·Pi species may be present if there are conformational intermediates before or after the hydrolysis step. It is possible that any or all of these species may be important in the polymerization reaction. Direct evidence for an actin·ADP·Pi was not obtained, presumably because the equilibrium of step 1 lies

far to the left.

(2) Covalently crosslinked actin dimer: The above experiments lead to the interesting hypothesis that the addition of an actin monomer to the end of an actin filament might best be represented as follows:



$$\begin{bmatrix} -(A\cdot D)_n A\cdot T \\ -(A\cdot D)_n A\cdot D\cdot Pi \\ -(A\cdot D)_n A\cdot D + Pi \end{bmatrix} + \begin{bmatrix} A\cdot T \\ A\cdot D\cdot Pi \\ A\cdot D + Pi \end{bmatrix} \longrightarrow \begin{bmatrix} -(A\cdot D)_{n+1} A\cdot T \\ -(A\cdot D)_{n+1} A\cdot D\cdot Pi \\ -(A\cdot D)_{n+1} A\cdot D + Pi \end{bmatrix} + Pi$$

i.e., any of three protomers at the end of a filament could interact with any of three monomer species for a total of 9 possible reactions at each filament end. For theoretical reasons, it is necessary that the release of Pi come from filament-bound ADP•Pi and not from monomer. Analyses of actin filaments show no detectable bound ATP or bound ADP•Pi but this is not unexpected because the protomers at filament ends represent a very small fraction of the total actin protomers in a filament. Analyses of actin filaments are analyses of the multiple internal actin protomers which would be expected to have only bound ADP. Therefore, we synthesized covalently crosslinked actin dimer by reaction of filaments of F-actin with phenylenebismaleimide, a reagent previously shown by others to crosslink actin into a mixture of polymerization-competent dimers and higher oligomers. After depolymerization, it was possible to isolate and characterize pure crosslinked dimer.

Actin dimer contains two moles of bound nucleotide and binds ATP much more tightly than it binds ADP. In this, it is more like G-actin than like F-actin. The exchange of dimer-bound ATP with free ATP is slower for both sites than it is for monomer actin but faster than for F-actin filaments. Exchange at one of the actin dimer sites is much more rapid than exchange at the other site and the site that exchanges ATP more rapidly contains about 15% of the bound nucleotide as ADP•Pi. The UV difference spectrum between monomer and dimer is very similar to the UV difference spectrum between monomer and polymer, suggesting that the dimer may be, at least partially, in the conformation of F-actin. These results suggest that the crosslinked actin dimer may be a useful model for one or both ends of the actin filament (the end are different) and that, as predicted, the ends may have a higher affinity for ATP than for ADP and may also contain bound ADP•Pi.

(3) Interaction of profilin with actin: Previous studies in this laboratory established that amoeba profilin, a 14,000-dalton protein, inhibits the rate of actin polymerization predominantly by inhibiting the nucleation reaction, the first step in polymerization, rather than the subsequent elongation of actin oligomers. Previous kinetic analysis of the stimulation by profilin of the rate of exchange of actin-bound ATP proved that a 1:1 molar complex formed between actin monomer and profilin and suggested a K_D of about 5×10^{-5} M for the reaction. This interaction has now been further studied.

Profilin has been found to inhibit the ATPase activity of actin monomer below its critical concentration. This observation agrees with the concept that the ATPase reaction might regulate actin polymerization and that proteins that regulate actin polymerization might do so by affecting the ATPase activity of actin.

It has also been possible to show for the first time the freely reversible, rapid equilibrium of the profilin-actin and actin-actin interactions. The amount

of polymerized actin, in the presence and absence of profilin, was determined as a function of actin and profilin concentrations by light scattering measurements. The same results were obtained beginning with G-actin as with F-actin. This establishes for the first time that profilin affects the elongation reaction as well as the nucleation reaction. The effects of profilin on nucleation are more dramatic only because the rate of nucleation is a function of the third power of actin concentration. From these studies a $K_D = 5\text{-}10 \times 10^{-6}$ M was calculated for the actin-profilin interaction, about one-tenth the previous value and one that would make profilin a more effective regulator of the polymerization process.

Proposed course of research: The ATPase activity of actin will be studied further in the hope that it will be possible to determine which of the several species of actin that are present during polymerization is the preferred species for addition to the elongating filament. Attempts will also be made to determine if the release of the filament-bound reaction product, Pi, occurs from the terminal protomers or from a population of protomers internal to the end. The crosslinked actin dimer will be studied further to characterize its interaction with cytochalasin (does it behave like actin polymer or actin monomer , its ability to interact with profilin (does it behave as actin polymer or monomer) and the hydrolysis of the bound ATP when actin is polymerized. The actin-profilin interaction will be further studied in terms of the binding site specificities and a more detailed analysis of the effect of profilin on the actin ATPase activity. Efforts will be initiated to study the association of actin filaments and actin monomers with plasma membranes of cells.

Publications:

1. Mockrin, S. C., and Korn, E. D.: Acanthamoeba profilin interacts with G-actin to increase the rate of exchange of actin-bound adenosine triphosphate. Biochemistry 19: 5359-5362, 1980.

2. Korn, E. D.: The polymerization of actin. In Schweiger, H. G. (Ed.): International Cell Biology 1980-1981. Springer-Verlag Press, 1981, pp. 336-345.

3. Korn, E. D.: Acanthamoeba castellanii: Methods and perspectives for study of cytoskeletal proteins. In: Wilson, L. (Ed.) Methods in Cell Biology "The Cytoskeleton". Vol. 23, part B, in press.

4. Mockrin, S. C., and Korn, E. D.: Isolation and characterization of covalently crosslinked actin dimer. J. Biol. Chem. 256, in press.

5. Brenner, S. L., and Korn, E. D.: Stimulation of actin ATPase activity by cytochalasins provides evidence for a new species of monomeric actin. J. Biol. Chem. 256, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00503-09 LCB

PERIOD COVERED
October 1, 1980 to September 30, 1981

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

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Other:	Nirbhay Kumar	Visiting Fellow	LCB NHLBI
	Jayasree Nath	Visiting Associate	LCB NHLBI
	Elliott Schiffmann		LDBA NIDR

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LAB/BRANCH
Laboratory of Cell Biology

SECTION
Organelle Biochemistry

INSTITUTE AND LOCATION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	3.0	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
We are studying a post-transitional modification of tubulin involving the reversible enzymatic addition of a C-terminal tyrosine. We have identified 4 ways the modification can modulate in vitro assembly: tyrosinolated tubulin bound more MAP-2, bound less phospho-MAP-2, assembled better with taxol, and formed shorter microtubules with MAP-2. In vivo, the apparent acceleration of tyrosinolation associated with leukocyte chemotaxis was shown not to be due to changes in the tyrosine pool; however in activated cells 2/3 of the tyrosine was replaced by an unknown catabolite. With HeLa cells we confirmed that tubulin tyrosinolated in vivo may be a structural variant from that which is a substrate in vitro. We have isolated a brain protein (unrelated to the tyrosinolation project) which specifically binds to and precipitates tubulin. It is an oligomer of a 50,000 dalton subunit distinct from any neurofilament or tau protein, and is uniquely resistant to phosphorylation. It might function to block indiscriminate assembly. It appears to bind less well in the presence of MAPS, but microtubules assembled in its presence were decorated with globular material.

PHS-6040
(Rev. 2-81)

Objectives: Our objectives are to ascertain the biological functions of a new tubulin binding protein, and of microtubule protein modifications by tyrosinolation and phosphorylation.

Methods Employed: Biochemical and cytological procedures as indicated under Major Findings.

Major Findings:

1. Detyrosinolating enzymes (N. Kumar): We reported last year that the principal brain enzyme that releases C-terminal tyrosine from tubulin (CPT, or carboxypeptidase-tubulin) acts preferentially on polymeric and oligomeric species of tubulin (in contrast, the enzyme that adds tyrosine acts preferentially on tubulin subunits). We have since further defined the specificity of our partially purified CPT fraction. Towards tubulin it had no endoprotease activity, or ability to digest further in to the C-terminus after releasing tyrosine. With respect to other substrates with C-terminal tyrosine, while it may not be completely specific for tubulin, it showed at least a 400 times greater preference than pancreatic carboxypeptidase A did for tubulin over muscle aldolase. The intriguing implication of these results is that there might be functionally important coupling between cycles of (de)-tyrosinolation and (dis)assembly. The finding that other carboxypeptidases does not show a preference for polymeric tubulin suggests that the chain C-terminus may not be simply more exposed and accessible in the polymer. Otherwise there is so far no evidence suggesting a functional coupling. An observation suggesting CPT specificity towards a subspecies, or restricted domain, of microtubules was that the increased rate, under assembly conditions, was manifest only while the first 1/3 of substrate was being consumed, although about 4/5 of the substrate was in microtubules. To test whether microtubule ends might be a favored domain, we compared rates of detyrosinolation or populations of microtubules with different average lengths, but could find no differences.

2. A New Tubulin Binding Protein (N. Kumar): In the course of purifying CPT we were led by chance to the discovery of a new and unrelated brain protein which binds specifically to tubulin. A protein fraction eluting from CM-cellulose just after CPT was found to precipitate pure tubulin (but not MAPS). SDS-PAGE of the precipitate chowed that one component of the fraction had very selectively bound to tubulin. The molecular weight of this TBP (tubulin binding protein) was about 50,000 daltons; non-denaturing PAGE suggests it may be an oligomer of 4 50K subunits. We have no stoichiometry, but pellets may contain as much TBP (by weight) as to be roughly comparable to the amount of a tubulin subunit. TBP does not correspond to any major neurofilament of Tau protein. It also could not be phosphorylated by a cAMP dependent kinase; every other detectable band in the CM-cellulose fraction was phosphorylated.

Addition of up to 0.3 parts by weight of TBP to tubulin (purified on phosphocellulose and assembly-incompetent) gave an instantaneous and proportional increase in turbidity, at 0° or 37°. Larger amounts caused gross coagulation. We have not detected any structure in these precipitates by EM. The precipitation was prevented (but not reversed) by 100 mM NaCl, or 5 mM ATP or GTP. The nucleotide effect was neutralized by equimolar Mg^{2+}. Podophyllotoxin did not compete with TBP, which is not therefore its physiological counterpart. However, one can envision a role for TBP in blocking indiscriminate assembly at the wrong times and places.

An alternate function in binding to and modifying the properties of microtubules is not ruled out, although so far it is less clear in what way TBP

interacts with microtubules, or tubulin in the presence of assembly-promoting MAPS. Addition of MAP-2 to tubulin at 0° (allowing formation of rings but not microtubules), diminished the turbidity elicited by subsequent addition of TBP at 0°, suggesting tubulin in rings may be less available to bind TBP. Warming then produced additional turbidity with kinetics characteristic of normal microtubule assembly. Negative staining showed microtubules plus amorphous material. Many microtubules appeared to be decorated with globular material, and some were suspended throughout their lengths above the grid coat surface. A few (1%) of these microtubules survived cold and Ca^{2+} disassembly.

3. Comparison of Tyrosinolated and Detyrosinolated Tubulin: Binding of Assembly Promoting Proteins, and Various Parameters of in vitro Assembly (N. Kumar): In the 6 years since the discovery of this post-translational modification of tubulin, it had not been shown to affect in vitro assembly. In the past year we have identified some differences. Last year's report described the preparation of pure tubulin by phosphocellulose (PC) chromatography after 3 assembly cycles, of detyrosinolated (DPC) tubulin with little or no C-terminal tyrosine, and of maximally tyrosinolated (TPC) tubulin with about 50% C-terminal tyrosine; in some experiments we also used PC-tubulin without further laboratory modification, which when isolated from brain has 10 to 20% C-terminal tyrosine. Under physiological conditions these tubulins assemble on the addition of crude MAPS fraction, or homogeneous MAP-2, or taxol.

DPC and TPC assembled to the same extent with different MAP-2 concentrations, and at saturating concentrations assembled with the same molar ratio of 1 MAP-2 to 9 tubulins in the pelleted microtubules. At a subsaturating concentration, the ratios wer 1:13 for TPC and 1:16 fot DPC. Thus TPC bound slightly more MAP-2. With saturating taxol (which we found to be the concentration equimolar to tubulin) DPC and TPC assembled at the same rate and to the same extent. But with limiting taxol TPC assembled about twice as fast and to a 2 times greater extent. Repeated with 4 batches of the modified tubulins, this is perhaps the most striking difference we observed. Lacking labeled taxol, we were not able to determine the amounts bound to DPC and TPC.

Phosphorylation of MAP-2 by a cAMP-dependent kinase has been variously reported to (a) have no effect on in vitro assembly, (b) to inhibit assembly, (c) to accelerate steady state treadmilling 30-fold. The following results indicate that phospho-MAP-2 is bound less effectively by TPC. When assembled with subsaturating MAP-2 in the presence of tracer amounts of ^{32}P-MAP-2, the microtubules formed from DPC and TPC contained equal amounts of ^{32}P. When the ^{32}P-MAP-s was added only after assembly had reached a steady state, 40% more ^{32}Pwas incorporated into the DPC microtubules. The incorporation of ^{32}P was more rapid than treadmilling, and appears to reflect equilibration of free MAP-2 into the intact microtubule.

In view of the recent discovery of phosphotyrosine in proteins, we did an additional experiment to test whether phosphorylation of the C-terminal tyrosine might be an intermediate step in MAP-2 phosphorylation. Using crude MAPS as both substrate and kinase, we found the rate of MAP-2 phosphorylation to be the same in presence of either tyrosinolated or detyrosimolated tubulin.

Using the ^{3}H-GTP exchange method, we found no significant difference in steady state treadmilling rates of microtubules formed from MAP-2 + DPC or TPC. However the rates varied greatly between batches of either.

Although we have not yet achieved the technique to compare MAP arm periodicities on microtubules, we compared 2 other ultrastructural features. Using thin sections of tannic acid stained pellets of microtubules formed from excess MAP-2 and DPC

or TPC, we found the average protofilament number to be exactly 12.6 in both cases. Average lengths of microtubules assembled with excess MAP-2 to steady state were found to be significantly different: for detyrosinolated tubulin (6% C-terminal tyrosine) the value was 6.54 μ, for unmodified PC tubulin (21%) the value was 5.80, and for TPC (42%) it was 4.72. The enhanced nucleation observed with TPC might correlate with its greater MAPS binding.

4. Problems Relating to the Characterization of the Species of Tubulin which is not a Substrate for Tubulin-Tyrosine Ligase (J. Nath): About half the tubulin from every cell or tissue so far examined is not a substrate for enzymatic tyrosinolation; more specifically it can not accept tyrosine even after digestion with pancreatic carboxypeptidase A (CPA) to remove whatever tyrosine was already present.

Last year we described a paradox involving the membrane-bound moiety of brain tubulin: it assayed as having no C-terminal tyrosine when extracted, yet in brain mince it fixed tyrosine as extensively as soluble tubulin did. The fixed tyrosine was all in α chain, and was all released as tyrosine (not a derivative) by CPA. The paradox was resolved as follows. The ligase reaction is reversible, releasing tyrosine in the presence of ADP + P_i, but is more specific in doing so than CPA or CPT (described above). Unlike the latter, it is inactive toward denatured tubulin, and therefore potentially selective toward native structural variants. We found in fact that ligase would not release tyrosine fixed in vivo in brain membrane tubulin. This tyrosinolated tubulin therefore is in the "non-substrate" moiety.

Further experiments with brain mince have shown that the soluble tubulin tyrosinolated in vivo is probably a mixture of substrate and non-substrate. One possibility is that ligase tyrosinolates tubulin in vivo, but another post-transitional modification follows rendering it non-substrate. In testing this, we found that substrate tubulin, ^{14}C-tyrosinolated with ligase in vitro, was not rendered non-substrate by incubation with partially homogenized mince, or when added to mince during homogenization.

Since much of a brain mince is damaged tissue, we are now pursuing the problem with cultured cell lines characterized by a high tubulin content. Tyrosine fixed in NG (neuroblastoma-glioma hybrid) cells could be substantially released by ligase. In contrast soluble tubulin tyrosinolated in HeLa cells appeared to be exclusively non-substrate. ^{3}H-Tyrosinolated tubulin formed by HeLa in vivo was mixed with brain tubulin ^{14}C-tyrosinolated in vitro, and the time course of release of the 2 labels was followed in presence and absence of ligase. The result showed unequivocally that the tubulins were not equivalent for ligase, and also suggested the presence of a new detyrosinolating enzyme with a preference for the non-substrate sample.

During the past year Kirschner's laboratory has sequenced a chick brain mRNA coding tubulin α chain, and shown that the terminal codon is for tyrosine. One must then consider that the first post-transitional modification may be removal of a coded tyrosine, which may later be added back. It is tempting to think that the 2 kinds of tubulin correlated in some way with the coded and post-transitional tyrosinolations.

5. Problems Relating to Tubulin Tyrosinolation in Living Cells (J. Nath): Using a mixture of antibiotics to block protein synthesis, we reported last year that the apparent rate of tyrosinolation of soluble tubulin in peritoneal leukocytes was increased by exposure of the cells to a chemoattractant, the peptide

formyl-met-leu-phe (fmlp). The increase was blocked by agents preventing the chemotactic response by affecting the following membrane phenomena: blocking the fmlp receptor, blocking Ca^{2+} influx, inhibiting phospholipase A2, inhibiting phospholipid methylation. In contrast cochicine, which prevents chemotaxis by dissolving the cytoskeleton rather than by affecting membrane phenomena, did not block the increase in tyrosine fixation.

In the past year four principle new results have been obtained with leukocytes.

(1) Taxol had recently been shown to stabilize or rapidly "freeze" cytoplasmic microtubules in several cell types. We found that taxol blocked chemotaxis, and markedly inhibited the rate of detyrosinolation to below the control level. So far the results with colchicine and taxol suggested tyrosine fixation was facilitated in proportion as tubulin was present as unassembled subunits. The effect of taxol on peptide-stimulated fixation gave a different picture. When taxol was added after preincubation with chemoattractant, stabilizing microtubules after they had had the opportunity to undergo cytoskeletal rearrangements involved a chemotaxis, tyrosine fixation was inhibited; conversely, when taxol was added first, presumably stabilizing the relatively small proportion of (inappropriately) assembled microtubules, subsequent addition of the chemoattractant was fully effective in stimulating fixation. These are not the results that would be expected if tyrosinolation were coupled, in a simple manner, to the distribution of tubulin between assembled and unassembled states.

(2) Since control leukocytes fix tyrosine without having been perturbed in any way that should shift the net extent of tubulin tyrosinolation, we had assumed that we were measuring turnover. We found, however, that when a 30-minute pulse of 10^{-8} M ^{3}H-tyrosine was followed by a chase of up to 1000 times higher concentration of unlabeled tyrosine, tritium fixation was arrested but nothing could be chased out. The result was buttressed by the observation that tyrosine could be chased in other cell types (see below). Since we calculate that labeled tyrosine has been fixed into up to 60% of the α chains without reaching a plateau, it seems quite unlikely that this could represent incremental net fixation.

(3) The specific radioactivity of the cellular pool of free tyrosine was determined under various conditions, to ensure that changes in it were not causing the apparent changes in rates of fixation. Leukocytes were preincubated for 30 minutes with protein synthesis inhibitors, then another 75 minutes with ^{14}C-tyrosine and: a) nothing (control); b) fmlp; c) 3-deazaadenosine + homocysteine; and d) taxol. The specific radioactivity of tyrosine had been replaced by an unknown tyrosine catabolite.

Related studies of other cell types showed that tyrosine fixed *in vivo* in a Trypanosome could be chased out; extracts of this protozoon also had ligase activity towards brain tubulin, in contrast to earlier and present negative results with Tetrahymena.

Other studies have been with HeLa and NG (neuroblastoma-glioma hybrid) cells, chosen because tubulin is easily purified due to its high concentration. The following correlations summarize the current status. a) In HeLa the substrate moiety of tubulin is completely tyrosinolated; in NG (like all other cells and tissues tested) less than half of this moiety is tyrosinolated. b) In HeLa all tyrosine fixed *in vivo* assays as non-substrate; in NG the majority can be released by ligase. c) In HeLa, tyrosine fixed *in vivo* can not be chased out; in NG 1/2 can be.

6. Miscellaneous Projects (M. Flavin): To begin to investigate the role of protein phosphorylation in microtubule assembly and function, we have purified calmodulin by fluphenazine-sepharose chromatography, and have prepared ^{32}P-histone and ^{32}P-MAP-2 to serve as substrates in a search for a MAP-2 specific phosphatase.

Among a flurry of recent papers on species and tissue variation in microtubule associated proetins, one reports that primates lack the MAP-1 and MAP-2 found in all other mammalian and avian brains. Since we had access to fresh (iced within 5 minutes of sacrifice) baboon brain, we examined this question by attempting to purify microtubule protein through assembly cycles. At each cycle the extent of both warm assembly and cold disassembly were extremely poor. In minute yield, the third warm pellet contained protein resembling MAP-2.

In collaboration with Dr. Alasdair Steven of NIAMDD we have been trying to make improvements in negative staining technique, in order to use optical diffraction to characterize MAPS periodicity in microtubules.

Proposed Course of Project:

Project 2: We want to find out whether this new tubulin binding protein functions to block indiscriminate assembly, or to modify the properties of microtubules. How much of it is there in cells, relative to tubulin, and with what stoichiometry do the two combine? We will want to work with a complex in solution, rather than a precipitate, then to purify the protein with a tubulin-sepharose column. Do tyrosinolated and detyrosinolated tubulin bind to it indiscriminately? Is it uniquely unavailable to the protein kinase because it already is fully phosphorylated? If this protein proves to be very specific for tubulin, and seems likely to have a physiological role, it will be desirable to raise antibodies against it for immunoassay and subcellular localization.

Projects 4 and 5: The focus of our long-standing interest in the biological function of tubulin modification by tyrosinolation seems at present to be on the nature of the "non-substrate" moiety. To try to correlate the coded with the non-substrate, we might study in vivo fixation of DOPA or phenylalanine; the ligase can fix these, the code could not. The puzzling inability to chase tyrosine from leukocytes and HeLa cells should perhaps be first approached by more careful analysis of free tyrosine pools during pulse and chase. EM studies of leukocytes treated with taxol may be the first step in examining the effects of this drug on stimulated tyrosine fixation.

Related Projects are the identification of the tyrosine catabolite in the amino acid pool of stimulated leukocytes, and the long-standing need to determine the subcellular localization of ligase and CPT.

Project 6: Since protein phosphorylation is a new project, we will briefly describe its current status in the microtubule field.

Map-2 is phosphorylated (3 mole/mole) by a cAMP dependent kinase tightly bound to it, perhaps via a regulatory subunit. It is also phosphorylated by a distinct, neurofilament associated kinase. Phosphorylation has been variously reported to have the following effects on in vitro assembly: no effect, inhibition, 30-fold stimulation of treadmilling rate.

Tubulin β chain from brain was reported 7 years ago to have 2 PSer residues,

a result which has not since been adequately confirmed or refuted. Recently a labile calmodulin-dependent kinase has been reported to phosphorylate the β chain in extracts. However the maximum extent observed was 0.01 mole/mole.

The role of calmodulin in *in vitro* assembly is unclear. One current report is that it enhances Ca^{2+}-liability in the presence (only) of MAPS, but to do so must be in 10-fold molar excess over tubulin.

We will start with the supposition that all the calmodulin effects are mediated by phosphorylations, and that conflicting results may point to multiple phosphorylations with opposing effects. Besides the cytoskeleton, we are also interested in phosphorylation of axonemal proteins, specifically in relation to ciliary arrest and reversal in Tetrahymena.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00506-06 LCB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Acanthamoeba myosins

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

PI:	Edward D. Korn	Chief	LCB NHLBI
Other:	Jimmy Collins	Staff Fellow	LCB NHLBI
	Graham Cote	Guest Worker	LCB NHLBI
	Joseph Albanesi	Staff Fellow	LCB NHLBI
	John Hammer	Staff Fellow	LCB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Section on Cellular Biochemistry and Ultrastructure

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.5	4.5	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)

Stable preparations of Acanthamoeba myosins IA and IB have been obtained which, for the first time, appear to be suitable for extensive physical chemical characterization of the differences between the phosphorylated (active) and non-phosphorylated (inactive) forms of the enzyme. Acanthamoeba myosin II contains three phosphorylation sites on each heavy chain that can be phosphorylated by a partially purified myosin II heavy chain kinase. In each case, a serine is phosphorylated. The three sites lie within a segment of about 3000 daltons very near the end of the tail of the heavy chain and about 100,000 daltons away from the ATPase site that they regulated. So far, only two of the three sites have been shown to be phosphorylated *in vivo* but this is sufficient to inactivate the enzyme completely. Actin-activatable myosin II (dephosphorylated form) shows stimulation by Ca^{2+} at 3 mM Mg^{2+} but not at 4 mM Mg^{2+}. It is inactive at less than 3 mM Mg^{2+}. Enzymatic activity shows indications of cooperative myosin-myosin interactions but there seems to be no correlation between enzymatic activity and filament formation.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: Previously we have shown that Acanthamoeba castellanii contains at least three myosin enzymes, each the product of a different structural gene. Myosin IA and IB have molecular weights of about 150,000 and each contains one heavy chain and two light chains (130,000, 17,000 and 14,000 daltons for IA; 125,000, 27,000 and 14,000 for IB). Myosin IA and IB can be phosphorylated by the same partially purified kinase which adds one phosphate specifically to the heavy chain. Only the phosphorylated forms of the enzymes show actin-activated Mg-ATPase activity. Neither light chain is required for enzymatic activity. The heavy chains can be digested with subtilisin and the phosphorylation site can be separated from the peptide that contains the ATPase site and retains actin-activated ATPase activity.

In contrast to these observations, myosin II is a more conventional myosin with a molecular weight of about 400,000 consisting of a pair of heavy chains of about 175,000 and two pairs of light chains of about 17,500 and 17,000. Myosin II can be isolated with either 1 or 2 moles of phosphate esterified to each heavy chain. The fully phosphorylated form shows no actin-activated Mg-ATPase activity but the partially phosphorylated form is actin-activatable. Dephosphorylation by commercial acid phosphatase converts both isolated forms of the enzyme into a very highly actin-activated myosin ATPase. Thus, this is the first myosin shown to be activated by dephosphorylation and Acanthamoeba is the first source of multiple myosin isoenzymes. Interestingly, myosin IA and IB seem to be preferentially localized near the cell surface and myosin II preferentially localized in the interior of the cell.

This year we had two major goals. (1) To attempt to make the difficult isolations of myosins IA and IB and the myosin I heavy chain kinase more routine. Then we would study the effect of phosphorylation on the physical parameters of the enzymes (in particular their abilities to form filaments) and the site of phosphorylation of the heavy chain (in order to determine the mechanism of derepression by phosphorylation). (2) We wanted to characterize further the enzymatic properties of dephosphorylated myosin II, to obtain the heavy chain kinase specific for it phosphorylation and to study the sites of phosphorylation relative to the ATPase site.

Methods employed and findings: (1) Myosin I. With slight modifications of the previous procedures we have been able to obtain, quite reproducibly, about 10 mg of myosin IA and IB from about 1 kg of cells. Although this is not much, it is sufficient. More importantly, conditions have been found which stabilize the enzyme so that, for the first time, it is possible to store preparations with neither loss of enzymatic activity nor proteolysis. The simplest procedure is just to freeze solutions of the enzymes at -20°, a procedure that works for these enzymes, but not for conventional myosins, presumably because myosins IA and IB are so much smaller. Preliminary evidence indicates that myosins IA and IB are monomers (both by electron microscopy and by sedimentation equilibrium) under conditions in which they are enzymatically active. But, of course, actin is not present in these determinations and it is possible that actin induces the polymerization of the myosins. The preparation of myosin I heavy chain kinase has also been improved but the enzyme is still not pure. It shows no activity, however, towards myosin II. All three of these enzymes (two myosins and one kinase) have been difficult to work with but their uniqueness makes them particularly interesting.

(2) Myosin II. The enzymatic activities of actin-activatable forms of myosin II have been characterized further. Myosin II is more highly activated by *Acanthamoeba* actin than by muscle actin. It is inactive below 3 mM $MgCl_2$ and fully active at 4 mM $MgCl_2$ and higher concentrations. Between 3 and 4 mM $MgCl_2$, micromolar Ca^{2+} fully activates the enzyme. Therefore, myosin II is the first myosin shown to have its actin-activated Mg-ATPase activity regulated both by the state of its phosphorylation and by micromolar Ca^{2+}. The activation by $MgCl_2$ is highly cooperative between 3 and 4 mM. However, assessment of filament formation by sedimentation shows that myosin II becomes sedimentable only at much higher concentrations of $MgCl_2$, about 6-7 mM, and that variations in the solubility do not parallel variation is enzymatic activity. Despite this evidence against filament formation as the basis of the cooperativity, the enzymatic activity as a function of myosin concentration also shows evidence of a cooperative interaction among myosin molecules.

A partially purified kinase has been prepared from *Acanthamoeba* which phosphorylates the heavy chain irrespective of the initial state of phosphorylation of the myosin. Extensive digestion of ^{32}P-myosin II with either trypsin or subtilisin generates the same three small radioactive peptides. In all three peptides, the phosphorylated amino acid is serine. Two of these three peptides are also labelled in peptide maps of myosin II isolated from amoebae grown on ^{32}Pi. It is not known if all three sites are labelled *in vivo*, and one is lost during isolation, or if the third site is an artifact of the *in vitro* phosphorylation. Kinetic correlation of the rate of phosphorylation of each of the three sites *in vitro* (beginning with dephosphorylated myosin II) and the loss of actin-activated Mg-ATPase activity indicates that it is sufficient to phosphorylate only the two sites that are phosphorylated *in vivo* to inactivate the enzyme completely. Those two sites probably act synergistically.

Limited digestion of the fully phosphorylated myosin II (3 labelled sites) has demonstrated that the three phosphorylation sites are all within about 3000 daltons of each other at, or very near, the tail of the heavy chain, and about 100,000 daltons away from the active site. This conclusion was reached as follows. Under appropriate conditions, incubation with chymotrypsin reduces the size of the heavy chain by about 9000 daltons, as judged by SDS gel electrophoresis, and simultaneously removes all the ^{32}P. Radioactivity can be recovered in a peptide of about 8500 daltons purified by gel filtration. Thus, all three sites lie within about 9000 daltons of one end of the heavy chain. Limited digestion with trypsin produces a peptide of about 112,000 daltons and a peptide of about 73,000 daltons. All of the ^{32}P is recovered in the 112,000-dalton peptide. When myosin II is digested after photoaffinity labelling with radioactive substrate, all of the covalently bound radioactivity is located in the 73,000-dalton peptide which, therefore, contains the active site and must be the head of the molecule. The 112,000-dalton peptide was isolated by HPLC in guanidine, renatured and shown to form filaments. Therefore, the phosphorylation sites are in the tail of the molecule and, from the chymotrypsin digestion, within about 9000-daltons of the end of the tail. Further trypsin digestion of the 112,000-dalton peptide releases all of the ^{32}P while reducing the size of the peptide by only about 3000 daltons. Therefore, the three phosphorylation sites lie within a segment of about 3000 daltons very near the tail of the molecule and at least 100,000 daltons away from the ATPase site which they regulate.

Proposed course of reasearch:

(1) We hope to be able to determine the state of association of myosin IA and IB in their phosphorylated and dephosphorylated forms to determine if there are any conditions in which these unusual myosins can be induced to make filaments. We will attempt to determine the nature of the change in the physical state of the enzymes that allows the phosphorylated form to be actin-activated. We will attempt to map the locations of the phosphorylation site relative to the ATPase site and to determine the location and sequence of phosphorylation sites.

(2) Similar approaches will be undertaken for the study of myosin II. Here we will attempt to determine if all three sites are phosphorylated by one enzyme or if the partially purified kinase preparation contains more than one enzyme, each with site specificity. We will attempt to isolate the 3000-dalton peptide that contains all three sites of phosphorylation and determine their precise relationships to each other. We will attempt to determine the physical changes induced in myosin II by phosphorylation in order to explain the nature of the enzymatic activation that occurs when the enzyme is dephosphorylated. We will attempt to purify from the Acanthamoeba the specific phosphatase that must be there in order to activate the enzyme *in vivo*.

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ANNUAL REPORT OF THE
LABORTORY OF CELLULAR METABOLISM
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

October 1, 1980 to September 30, 1981

Research in the Laboratory of Cellular Metabolism is largely concentrated on the enzymes responsible for the synthesis and degradation of cAMP and cGMP through which many hormones, drugs, and other agents influence cellular functions. In addition, studies of the regulation of lipid metabolism as well as investigations of the inflammatory process and histamine are continuing.

1. Cyclic Nucleotide Phosphodiesterases

This year, much of this work has been focussed on the calmodulin-activated phosphodiesterase. An improved rapid method for purification has been developed, which greatly facilitates acquisition of enzyme in amounts needed for structural studies, etc. These preparations have specific activities as high or higher than any reported, appear homogeneous in the analytical ultracentrifuge, and contain a single peptide of 58,000 daltons. The molecular weight of the native enzyme based on sedimentation equilibrium is 128,000. Extending earlier observations, we found that the enzyme in fresh supernatant behaved as a molecule of $M_r \sim 60,000$ but after 2 days its chromatographic behavior was that of a molecule of $M_r \sim 135,000$. Calmodulin stimulation of activity of the "aged" sample was about twice that of the fresh sample, indicating the time-dependent formation of enzyme dimers with a concomitant increase in the dependence of activity on calmodulin. Storage of purified enzyme at 4° led to a rapid loss of calmodulin stimulation but not of total activity along with conversion from a predominantly oligomeric to monomeric form. There was no evidence of proteolytic degradation and the enzyme was still quantitatively bound to and eluted from calmodulin-Sepharose. We concluded that loss of calmodulin responsiveness was related to the appearance of monomeric enzyme, which can interact with but is not further stimulated by calmodulin.

The relationship of subunit structure to catalytic activity was further investigated using target theory analysis of data from radiation inactivation experiments carried out with scientists in MDB, NHLBI, and LPB, NIADDK. Low doses of radiation caused a 10 to 15% increase in basal activity, which, with further irradiation, decayed with an apparent target size of $\sim$ 60,000 daltons. Calmodulin-dependent activity decayed with an apparent target size of $\sim$ 105,000 daltons. The percentage stimulation of enzyme activity by calmodulin decreased markedly as a function of radiation dosage. These observations are consistent with the predictions of computer-assisted modeling based on the assumptions that: 1) The calmodulin-activated phosphodiesterase exists as a mixture of monomers that are fully active in the absence of calmodulin and dimers which are inactive in the absence of calmodulin. 2) In the presence of calmodulin, a dimer exhibits activity equal to that of two monomers. 3) On radiation destruction of a dimer, an active monomer is generated. This monomer-dimer hypothesis provides for the first time a plausible explanation for and definition of basal and calmodulin-dependent phosphodiesterase activity.

In collaborative experiments, we investigated the interaction of the purified phosphodiesterase with a fluorescent derivative of calmodulin, dansyl calmodulin. On binding Ca^{2+}, the fluorescence maximum of dansyl calmodulin was shifted

from 525 to 490 nm and fluorescence intensity was increased. Addition of the phosphodiesterase or other calmodulin-binding protein (troponin I or modulator-binding protein) caused a further "blue shift" and increase in intensity. Measurements of fluorescence polarization also demonstrated the interaction of dansyl calmodulin and phosphodiesterase in the presence, but not in the absence, of Ca^{2+}. From other experiments, we obtained evidence that the affinity of dansyl calmodulin for Ca^{2+} is increased when it is associated with the phosphodiesterase and seemingly represents an important regulatory property of calmodulin.

For study of the physiological regulation of phosphodiesterase activity, at present poorly understood, cultured cells offer many advantages. Hepatoma cells were successfully used in our earlier work to demonstrate mechanisms for the independent modulation of cGMP and cAMP phosphodiesterases. These cells, however, lack the calmodulin-activated phosphodiesterase, and nothing is known about factors that might influence its activity. We have, therefore, initiated studies with cultured fibroblasts to investigate the effects of glucocorticoids and other hormones on this enzyme. We are continuing attempts to prepare monoclonal antibodies to the phosphodiesterase, which should be useful in some of these experiments as well as for defining functional domains in the isolated enzyme and comparing the antigenic properties of different phosphodiesterases.

2. Adenylate Cyclase

Although much of our work in this area continues to involve choleragen, we have recently increased efforts to separate and purify the individual components of the adenylate cyclase system, i.e., hormone receptor, guanine nucleotide-binding protein (G/F), and catalytic unit (C). In addition, studies of NAD:arginine ADP-ribosyltransferases in animal cells are summarized here as they were discovered in our search for enzymes analogous to choleragen that might be involved in the physiological regulation of adenylate cyclase activity.

The A_1 peptide of choleragen activates adenylate cyclase only after it is released from the holotoxin by reduction of the disulfide bond that links it to A_2. It is not known whether in intact cells this reduction is enzymatic or nonenzymatic. In collaboration with J. E. Dixon, Purdue University, we have shown that purified thiol:protein disulfide oxidoreductase from bovine liver increases the rate of choleragen activation in the presence of limiting concentrations of glutathione or dithiothreitol and decreases the concentration of thiol required for activation. As this enzyme is found in plasma membranes of many cells, it could play a role in choleragen action. Numerous studies have implicated GTP in the activation of adenylate cyclase by choleragen, but its precise role was unclear. We, therefore, investigated nucleotide effects on 1) choleragen activation of adenylate cyclase, 2) thermal stability of the activated enzyme, and 3) its catalytic activity. All were dependent on GTP; ITP and ATP were less effective. It is concluded that GTP, in micromolar concentrations, is important not only in the activation of adenylate cyclase by choleragen but also in the stabilization and expression of catalytic function of the activated enzyme.

Activation of adenylate cyclase by choleragen is believed to result from ADP-ribosylation of G/F. Others have shown that purified G/F contains two peptides that are choleragen substrates, and ADP-ribosylation of one or two peptides of similar size in several membrane preparations has been reported. Because choleragen can ADP-ribosylate so many unrelated proteins, however, it seemed important to determine which proteins are choleragen substrates in intact cells. We

reasoned that peptides ADP-ribosylated during incubation of cells with choleragen might no longer serve as ADP-ribose acceptors and, therefore, would not be labeled when cell membranes were subsequently incubated with toxin and [^{32}P]NAD. In membranes from cultured fibroblasts, choleragen ADP-ribosylates peptides of 42,000 and 47,000 daltons. When cells were incubated with choleragen before preparation of membranes, no labeling of these proteins was observed. The extent of the decrease in labeling paralleled the magnitude of the effect of the toxin on adenylate cyclase activity as the latter was varied with time, choleragen concentration, or antitoxin. Labeling was not decreased when cells were exposed to choleragen A or B subunits, which do not activate adenylate cyclase, or to epinephrine or prostaglandin E_1, which activate adenylate cyclase and through a mechanism different from that of the toxin. Thus, all of the data are consistent with the conclusion that the 42,000 and 47,000 dalton peptides, presumed components of G/F, are the natural substrates for choleragen in intact fibroblasts.

In the current view, C is catalytically active when associated with G/F that has bound GTP; hydrolysis of GTP to GDP results in an inactive complex, and hormones activate adenylate cyclase by promoting the replacement of GDP by GTP. There is still, however, lack of agreement concerning how ADP-ribosylation by choleragen alters G/F function, i.e., whether GTP hydrolysis is inhibited or replacement of GDP is accelerated. Isoproterenol is known to stimulate release of bound GDP from turkey erythrocyte membranes, and we have now found that choleragen does likewise. Our data are consistent with the conclusion that both agents release GDP from the same sites. Thus, choleragen, as well as isoproterenol, may activate the cyclase by decreasing the affinity of G/F for GDP. Understanding the nature of the interaction between G/F and C will require finally reassembly of the separately purified proteins into functional complex. In undertaking to purify the two components, we have found that solubilization of adenylate cyclase with CHAPS, a new synthetic zwitterionic detergent, offers several advantages over previously described procedures. Extracts of high specific activity are obtained and, in the presence of CHAPS, G/F and C are readily separated by gel filtration. Although the instability of C remains a problem, we have recently learned that ATP and App(NH)p (but not several other nucleotides) retard the thermal inactivation of C. This is of interest not only because of its potential usefulness in the purification of C but also because the nucleotides are effective in the absence of divalent cations, whereas the substrate for C is Mg^{2+}- (or Mn^{2+}-) nucleotide.

The activity of the NAD:arginine ADP-ribosyltransferase that we have purified from turkey erythrocytes is enhanced by several inorganic anions, their order of effectiveness corresponding to the Hofmeister series. Based on kinetic data and physical properties of the purified transferase, we conclude that these salts favor dissociation of relatively inactive enzyme oligomers to protomers with high catalytic activity. In the absence of salt, histones can stimulate ADP-ribosylation of agmatine $\sim$ 10-fold to a level approaching the maximum obtained with NaCl. Histones, which do not alter activity when the salt concentration is optimal, are significantly more effective than other proteins tested. Maximal activation is produced with < 1 μM histone, whereas much higher concentrations are required for the protein to serve as a substrate. It appears that the active forms of the enzyme may be identical, whether generated in the presence of salt or histones, and the latter may serve as physiological effectors modulating intracellular transferase activity. We have recently purified an NAD:arginine ADP-ribosyltransferase from human erythrocytes that resembles the avian enzyme in many ways including activation by histones or salt and have found similar enzymes in

other animal tissues consonant with the view that ADP-ribosylation may be an important cellular regulatory mechanism.

3. Guanylate Cyclase Activity

From studies in several laboratories, it has been inferred that sulfhydryl groups could play a role in guanylate cyclase activity. Using extensively purified preparations of the soluble enzyme from rat and calf liver, we now have directly demonstrated the temperature-dependent inactivation of guanylate cyclase by compounds capable of forming mixed disulfides or covalent mercaptide bonds. Inactivation produced by GSSG was rapidly reversed by GSH or cysteine as well as by dithiothreitol which also reversed that produced by other compounds. Based on kinetic studies with diamide or GSSG, we conclude that reaction with a single sulfhydryl group on the enzyme leads to inactivation. Although some inactivation experiments were carried out in the presence of the guanylate cyclase activator, which was necessary for stabilization of the dilute enzyme (particularly at 30°C), reversible inactivation by p-hydroxymercuribenzoate at 0°C was also demonstrated in the absence of activator. In addition, exposure of activator to GSSG or dithionitrobenzene did not alter its ability to enhance guanylate cyclase activity. Thus, it appears that disulfides and sulfhydryl reactive compounds alter the activity of the enzyme itself, not that of the activator. The fact that inactivation by GSSG was decreased in the presence of Mn^{2+} and guanosine 5'-(β-,γ-imino)triphosphate, a GTP analog and competitive inhibitor of the enzyme, is consistent with this conclusion. Guanylate cyclase activity was reduced in soluble fractions from homogenates of rat liver slices previously incubated with agents expected to decrease in different ways the intracellular content of GSH and other sulfhydryls. Activity was restored by addition of DTT or GSH to assays. We believe that guanylate cyclase activity in intact cells may be subject to regulation through reversible modification of critical sulfhydryl groups, e.g., by protein thiol:disulfide exchange. To aid in further characterization of the regulatory properties of the guanylate cyclase as well as for studies of factors that may control the amounts of enzyme in cells, we have attempted this year to prepare monoclonal anbitodies. A recent hybridization of spleen cells from an immunized mouse with myeloma cells appears to be yielding several antibody-producing hybridoma, which are now being cloned.

4. Regulation of Lipid Metabolism in Mammalian Cells

Last year, we initiated investigation of the effects of carnitine on fatty acid metabolism in cultured fibroblasts and skeletal muscle cells from patients with a myopathy characterized histologically by the accumulation of lipid droplets in skeletal muscle cells; the carnitine content of the muscle is low and the syndrome has been termed "carnitine deficiency." In early experiments, high concentrations of carnitine were required to stimulate fatty acid oxidation and effects were small. Using cells maintained in medium containing serum or embryo extract exhaustively dialyzed to remove carnitine, we are now able to show effects of submicromolar carnitine. Although oxidation of other substrates was not impaired, fatty acid oxidation by carnitine-depleted cells was very low. Significant increases were observed with 25 nM carnitine and maximal increases (six- to tenfold) with 100 μM. In addition, incorporation of fatty acids into di- and triglycerides was elevated in carnitine-depleted cells which may explain the lipid deposition in muscles of patients with carnitine deficiency. Glucocorticoids have been used with apparent success for treatment of some of these patients. Dexamethasone enhanced fatty acid oxidation in fibroblasts and muscle

cells in the presence or absence of carnitine. Thus far, however, we have detected no differences between normal and patient fibroblasts in their responses to carnitine or dexamethasone.

Cholesterol synthesis in many mammalian cells is controlled by the activity of hydroxymethylglutaryl Coenzyme A (HMGCoA) reductase. Negative feedback regulation of this enzyme by cholesterol and low density lipoproteins (LDL) in cultured human fibroblasts has been extensively studied by several groups including our own. Hepatic HMGCoA reductase can, in addition, be rapidly inactivated by phosphorylation and reactivated by dephosphorylation. In collaboration with MDB, NHLBI, we have now shown for the first time that this regulatory mechanism is also operative in the cultured fibroblasts. After prolonged incubation of cells in the absence of serum lipids, HMGCoA reductase activity is high, and all of the enzyme was dephosphorylated. Following exposure of cells to LDL for one hour, $\sim$ 35% of the enzyme was phosphorylated, i.e., was inactive and was activated by dephosphorylation. By four hours, activity was decreased $\sim$ 90% as expected, presumably due entirely to a decrease in the amount of enzyme protein as all of the enzyme was dephosphorylated. Similarly, mevalonic acid, the first committed intermediate in the pathway of isoprenoid synthesis, caused reversible inactivation (phosphorylation) within 30 min and, by 60 min, reduction of activity not attributable to phosphorylation. Thus, in addition to their previously described relatively slow effects on HMGCoA reductase in cultured fibroblasts, mevalonate and LDL can produce more rapid and potentially reversible inactivation of the enzyme. Demonstration of the phosphorylation-dephosphorylation mechanism for regulation of HMGCoA reductase activity means that these cells may prove as useful for investigation of this system as they have in studies of the chronic regulation of enzyme levels.

5. Role of Histamine in Physiologic and Pathologic Reactions

It is estimated that $\sim$ 10% of the population suffers from some type of allergic reaction during a year and for $\sim$ 0.1% the consequences may be severe or fatal. Our studies of factors involved in histamine synthesis and storage in mast cells and the physiological mechanism for its degradation after release from mast cells are directly related to this problem. In addition to its involvement in pathological reactions, histamine has a physiological role in the central nervous system and in the regulation of gastric secretion as shown by work in several laboratories including our own. Our continuing collaboration with scientists at the V. A. Hospital in Los Angeles has yielded much new information about the precise location of histamine in the gastric gland (mast cells in dog and man vs. enterochromaffin-like cells in rodents) and the distribution of histamine-metabolizing enzymes within the gastric mucosa.

We have shown that intact mast cells from several organs of rats and dogs transport histidine through a system (N) that also transports glutamine. Uptake of radiolabeled histidine (and synthesis of histamine from it) was blocked by glutamine and several histidine analogs, i.e., α-methylhistidine, 2-fluorohistidine, and β-thienylalanine. The latter two should prove useful in further studies of the transport system. Histamine synthetic activity was largely lost upon cell disruption, for reasons that are not clear, but not upon degranulation of cells induced by Compound 48/80. Thus, histidine decarboxylase appears to be located in the cytosol rather than in the histamine-containing granules.

In dogs, the infusion of histamine at rates that increase gastric secretion to 20 to 75% of the maximum rate increased arterial plasma histamine levels 0.5 to 3 μM. Based on arteriovenous differences, there was complete clearance of histamine by the hind limbs and kidney and partial clearance (perhaps due to shunts) across the gastric, hepatic, and intestinal beds. After termination of histamine infusion, arterial plasma levels declined rapidly ($t_{1/2} \sim 25$ s). We have begun to investigate the possible role of vascular endothelial cells in this process. Activities of the histamine-inactivating enzymes, histamine methyltransferase and diamine oxidase, were high in microvessels from several organs of rats and guinea pigs. Microvessels from fat pads accounted for almost all of the enzyme activities of the whole tissue. Methyltransferase was equally high in microvessels from heart and in cardiac muscle cells. Preliminary experiments indicate that histamine uptake by microvessels resembles in many ways serotonin uptake by these cells.

6. Mechanism of Action of Anti-Inflammatory Drugs

Recent research on the nonsteroidal anti-inflammatory agents has focused on their ability to inhibit production of arachidonic acid metabolites, particularly those of the cyclooxygenase pathway. We have investigated the cytostatic and immunosuppressive activities of these drugs, however, as these may also be of therapeutic importance. In our earlier studies, all of the common anti-inflammatory drugs reversibly arrested growth of several types of cultured cells in the G_1 phase of the cell cycle. The cytostatic effects, paralleled the anti-inflammatory activity of the drugs, although they apparently did not result from inhibition of prostaglandin synthesis. Others have reported that anti-inflammatory drugs inhibit blast transformation of lymphocytes at an early stage of the process. As in the cultured cells, the cell cycle is not interrupted when drugs are added after DNA synthesis has started. We feel that the anti-inflammatory drugs act through similar mechanisms in cultured cells and lymphocytes, possibly by impairing the coupling of energy-producing system(s) to amino acid transport. Studies on several transport systems in cultured and freshly isolated cells were completed this year. Transport of glucose, nucleotides, and neutral amino acids through nonenergy-dependent N, ASC, and L systems is not inhibited by anti-inflammatory drugs, whereas the energy-dependent A system for amino acid transport is inhibited as reported last year. The capacity (V_{max}) but not the affinity of the A system is markedly increased as cultured cells enter exponential growth and following the addition of mitogenic agents to lymphocytes when the increase is observed before other changes (in cyclic AMP, polyamine, or DNA synthesis) are evident. In all of the cells, enhancement of amino acid uptake is prevented by anti-inflammatory drugs. As noted last year, the affinities for Na^+ and amino acid are not altered, only V_{max}. After removal of the drug, transport increases followed by initiation of DNA synthesis and mitosis. Thus, the changes in amino acid transport appear to reflect changes in the number of functional carriers for the A system and to be related to cell growth and mitosis.

We are continuing to investigate effects of anti-inflammatory drugs on two types of inflammatory responses. The first, observed after burn injury or produced experimentally by the intrapleural injection of dextran, is characterized by degranulation of mast cells and rapid effusion of protein-free fluid; it is not affected by anti-inflammatory drugs. The second type is exemplified by the reaction to intrapleural carrageenan in which there is a progressive accumulation of fluid, plasma proteins, neutrophil chemotactic activity, and neutrophils.

Accumulation of all four is suppressed to the same extent by indomethacin. It appears that indomethacin suppresses the carrageenan-induced leakage of serum proteins into the pleural space but does not interfere with the subsequent generation of chemotactic activity, which may result from activation of the complement system.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00603-07 CM

PERIOD COVERED
October 1, 1980 through September 30, 1981

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:	Irene E. Stith	PRAT Fellow	CM	NHLBI
	Vincent C. Manganiello	Head, Section on Biochemical Physiology	CM	NHLBI
	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI

COOPERATING UNITS (if any)
Dr. Stith is in the Pharmacology/Toxicology Program, National Institute of General Medical Sciences, NIH

LAB/BRANCH
Cellular Metabolism

SECTION
Metabolic Regulation

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.2	PROFESSIONAL: 1.2	OTHER: 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)

Soluble cyclic GMP phosphodiesterase activity is present in cultured fibroblasts. Activity is stimulated as much as 3-fold by calcium and calmodulin. The amount of cyclic GMP phosphodiesterase activity detected is dependent upon the methods used to harvest the cells and to prepare subcellular fractions. Homogenization of cells substantially reduces cyclic GMP phosphodiesterase activity and calmodulin-sensitive activity. Harvesting cells by scraping and centrifugation at 10,000 x *g* or 100,000 x *g* reproducibly yields a supernatant fraction which contains 90% of the total cyclic GMP phosphodiesterase activity and calmodulin-sensitive activity detected before centrifugation.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: For study of the physiological regulation of cyclic nucleotide phosphodiesterase activity, which is at present poorly understood, cultured cells offer many advantages. Hepatoma cells were successfully used in earlier work in this laboratory to demonstrate mechanisms for the independent modulation of cGMP and cAMP phosphodiesterases. These cells, however, lack the calmodulin-activated phosphodiesterase, and nothing is known about factors that might influence its activity. We have, therefore, initiated studies with cultured fibroblasts to investigate the effects of glucocorticoids and other hormones on this enzyme.

Major Findings: Initially, it was necessary to establish a suitable procedure for harvesting fibroblasts and preparation of subcellular fractions for phosphodiesterase assay. Avoiding proteolysis is particularly critical when working with calmodulin-activated enzyme as limited proteolysis can cause irreversible activation with loss of calmodulin responsiveness. The use of trypsin to remove the cells from culture dishes was, therefore, ruled out. It was found that scraping cells with a rubber policeman released virtually all malic dehydrogenase activity into the medium, and following this treatment no viable cells were detected. After centrifugation of the suspension of scraped cells, $> 90\%$ of the cGMP and cAMP phosphodiesterase activity was found in the soluble fraction. Homogenization of the suspension before centrifugation decreased the recovery of activity. In addition, the magnitude of the calmodulin effect was extremely variable in such preparations, whereas in supernatants prepared without homogenization of cells calmodulin increased activity $\sim 300\%$. Fractions prepared in this way contain $> 90\%$ of the total, as well as the calmodulin-activated cGMP phosphodiesterase.

A method for separation of the calmodulin-activated phosphodiesterase from others present in the fibroblast supernatant (following removal of calmodulin on an ion-exchanger) using calmodulin-Sepharose affinity columns has been validated, and necessary control data on the levels of phosphodiesterase activity as a function of time in subculture are being accumulated.

Significance to Biomedical Research: Knowledge of properties of the cyclic nucleotide phosphodiesterases and their regulation is necessary to understand mechanisms for control of cAMP and cGMP content in cells, e.g., in cardiac muscle and lung.

Proposed Course: The effects of glucocorticoids and of agents that induce prolonged elevation of intracellular cAMP on activity of the calmodulin-activated phosphodiesterase will be investigated.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00606-10 CM

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Regulation of cAMP Content of Cultured Cells

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

PI:	Vincent C. Manganiello	Head, Section on Biochemical Physiology	CM	NHLBI
	Joel Moss	Head, Section on Molecular Mechanisms	CM	NHLBI
OTHER:	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI

COOPERATING UNITS (if any)
Fusao Hirata, Donna Bareis, and Julius Axelrod, Laboratory of Clinical Science, NIMH

LAB/BRANCH
Cellular Metabolism

SECTION
Biochemical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.7	0.7	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The cAMP content of cultured human fibroblasts is increased by incubation of cells with several effectors, e.g., bradykinin, PGE_1, isoproterenol, and choleragen. Bradykinin increases production of prostaglandins of the E series and prostacyclin, as measured by radioimmunoassay. Both prostaglandins, in turn, stimulate production of cAMP in cultured human foreskin fibroblasts. Responsiveness to bradykinin is altered by duration of incubation of fibroblasts in serum containing growth medium and by exposure of fibroblasts to agents such as dexamethasone, glutamine, and tryptophan. The effects of these agents can be related to their effects on bradykinin stimulation of prostaglandin production.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: To elucidate the mechanisms through which several hormones, other physiological "messengers" and drugs influence the synthesis and degradation of cyclic nucleotides in cultured cells. Cultured cells serve as excellent model systems of relatively homogeneous populations of cells which can be grown and studied under defined conditions.

Methods Employed: Cultured cells grown under standard conditions; cAMP and cGMP purified by techniques developed in the laboratory and assayed by commercially available radioimmunoassay, prostaglandins assayed by commercially available radioimmunoassays.

Major Findings: Mechanism of Action of Bradykinin. As reported last year, in human foreskin fibroblasts, bradykinin increases phospholipid methylation, alters Ca^{++} flux, promotes release of arachidonic acid from phospholipid, and increases prostaglandin synthesis and accumulation of cAMP. Inhibition of phospholipid methylation is associated with only partial inhibition of arachidonate release and prostaglandin synthesis. Inhibitors of phospholipid methylation do not alter effects of bradykinin on cAMP accumulation in the presence or absence of phosphodiesterase inhibitors. In fibroblasts incubated with labeled arachidonic acid, bradykinin enhanced release of arachidonate from both phosphatidylcholine and phosphatidylinositol. These data suggest that bradykinin activates phospholipases (A_2 and C) or certain "pools" of phospholipases by mechanisms independent of phospholipid methylation.

Stimulation of fibroblasts with bradykinin causes release of prostaglandins of the E series and prostacyclin, as measured by radioimmunoassay. Both prostaglandins increase accumulation of cAMP. In cells incubated for at least one week without change in serum-containing growth medium, bradykinin stimulation of prostaglandin production and, therefore, cAMP accumulation is decreased. Responsiveness to bradykinin in terms of prostaglandin production and cAMP accumulation is partially restored by incubation for 24 hr with tryptophan, glutamine, or with fresh serum-containing growth medium.

Incubation of cells with dexamethasone for 24-48 hr or indomethacin for 10 min also inhibits the effect of bradykinin on prostaglandin production and cAMP accumulation. Thus, the effects of alterations in growth medium and various agents on bradykinin-induced changes in cAMP content can be related to their effects on the stimulation of prostaglandin production by bradykinin.

Mechanism of Action of Lipomodulin. Lipomodulin, a protein which inhibits the action of phospholipase A_2 and C, has been found in cultured human foreskin fibroblasts. Lipomodulin content (in cells plus medium) is increased in cells exposed to dexamethasone. Incubation of cells with lipomodulin inhibits bradykinin-induced arachidonate release. Antibodies directed against lipomodulin enhance the release of arachidonate from fibroblasts and prevent the inhibitory effect of this protein on bradykinin stimulation of arachidonic acid release.

Significance to Biomedical Research: The actions of a number of hormones, neurotransmitters, and other humoral agents are thought to be mediated by their effects on cyclic nucleotide metabolism. Various effectors activate adenylate

cyclase by different mechanisms and fibroblasts represent a good model system to study the coordinate plus independent regulation of the responsiveness of the cyclase system to hormones and other humoral agents.

Bradykinin, a potent stimulus for prostaglandin production in many cell types, is thought to play an important role in maintenance of vascular tone and permeability and in inflammatory processes. The extent to which many of the actions of bradykinin are mediated by prostaglandins is at present unknown. Fibroblasts may constitute a good model system to study initial events in bradykinin action and in regulation of prostaglandin metabolism.

Proposed Course: Continued study of regulation of cAMP and cGMP content by bradykinin and other effectors as well as the mechanism of action of bradykinin.

Publications: Moss, J., Manganiello, V.C., Hom, B.E., Nakaya, S., and Vaughan, M: Effects of d- and l-propranolol on the responsiveness of human fibroblasts to choleragen and prostaglandin E_1. Biochem. Pharmacol. 30: 1263-1269, 1981.

Hirata, F., del Carmine, R., Nelson, C.A., Axelrod, J., Schiffmann, E., Warabi, A., De Blas, A.L., Nirenberg, M., Manganiello, V., Vaughan, M., Kumagai, S., Green, I., Decker, J.L., and Steinberg, A.D.: Presence of autoantibody for phospholipase inhibitory protein, lipomodulin, in patients with rheumatic diseases. Proc. Natl. Acad. Sci. U.S.A. 78: 3190-3194, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00607-08 CM

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Cyclic GMP Metabolism

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

PI:	Su-Chen Tsai	Research Chemist	CM	NHLBI
	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI
OTHER:	Vincent C. Manganiello	Head, Section on Biochemical Physiology	CM	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Cellular Metabolism

SECTION
Metabolic Regulation

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 2.2	PROFESSIONAL: 1.2	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)

Guanylate cyclase activity was reduced in soluble fractions from rat liver slices incubated with diamide, N-ethylmaleimide, or 5',5'-dithiobis-2-nitrobenzoate; addition of GSH or dithiothreitol to assays restored activity. Inactivation of purified rat liver guanylate cyclase by p-hydroxymercuribenzoate at 0°C was completely reversed by dithiothreitol. p-Hydroxymercuribenzoate and GSSG caused reversible inactivation of calf liver guanylate cyclase. Dithiobisnitrobenzoate and cystamine were more potent than GSSG; all were more effective at 30°C than at 0°C. It appears that guanylate cyclase activity in intact cells could be modulated by reversible modification of critical sulfhydryl groups, e.g., by thiol:disulfide exchange involving glutathione. Fusion of spleen cells from immunized mice with myeloma cells has yielded some hybrids that appear to be producing antibodies to the guanylate cyclase and these are being cloned.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: To purify guanylate cyclase and elucidate the mechanisms that regulate its activity.

Methods Employed: Guanylate cyclase is assayed in a system containing 1 mM [α-^{32}P]GTP, 5 mM cGMP, 6 mM theophylline, 50 µg of activator, and Tris-Cl buffer, pH 7.4. [^{32}P]cGMP was purified through columns for radioassay. The cyclase from liver supernatant is purified using salt fractionation, ion exchange, gel filtration, GTP-affinity chromatography, and electrophoresis. ^{125}I-guanylate cyclase is prepared by the method of Bolton-Hunter.

Major Findings: Reversible Inactivation of Purification and Characterization of Hepatic Guanylate Cyclase by Disulfides. We reported last year results of preliminary experiments on inhibition of guanylate cyclase by p-hydroxymercuribenzoate (PHMB) or oxidized glutathione (GSSG) and reactivation by dithiothreitol (DTT). These studies have been continued with rat liver guanylate cyclase. The highly purified enzyme from GTP-Agarose was rapidly inactivated by 200 µM PHMB at 0°C and completely reactivated with DTT. Although activities were higher when activator (see below) fraction was present in assays, the relative effects of PHMB and DTT were similar. Incubation of activator fraction with GSSG (or DTNB) did not alter its capacity to increase guanylate cyclase activity. GSSG as well as PHMB also inactivated purified calf liver guanylate cyclase. Inactivation persisted after removal of GSSG on Sephadex G-25; 2 mM DTT, GSH, or cysteine produced maximal reactivation, whereas ascorbate and nitroprusside had no effect. DTNB and, to a lesser extent, cystamine were more potent than GSSG in inactivating guanylate cyclase; all were more effective at 30°C than at 0°C. Inactivation by lipoic acid required relatively high concentrations and was seemingly less dependent on temperature. Inactivation produced by cystamine (up to 250 µM) or DTNB (up to 50 µM) was completely reversed by DTT, that by lipoic acid only partially.

Diamide was used to investigate the number of SH group(s) involved in guanylate cyclase inactivation. Kinetic analysis of the data indicated that the reaction of one mole of diamide with one mole of enzyme SH resulted in inactivation. Studies with GSSG as the inhibiting agent led to the same conclusion. Gpp(NH)p, a GTP analog and competitive inhibitor of guanylate cyclase, in the presence of Mn^{2+}, protected the enzyme from inactivation by diamide or GSSG.

Reversible Inactivation of Guanylate Cyclase in Liver Slices. Incubation of rat liver slices with 20 mM diamide, which has been reported to oxidize intracellular GSH to GSSG, for 20 min decreased guanylate cyclase activity assayed in the soluble fraction of homogenates by ∿ 50%. Activity was restored to levels equivalent to those from control slices by inclusion of 20 mM GSH in the assay; DTT was less effective than GSH. Similarly, activity from slices incubated for 5 min with 5 mM DTNB or 4 mM NEM was decreased ∿ 50%, and this inactivation was reversed by GSH or DTT. In most experiments, DTT and GSH had little effect on the activity from control slices, although they sometimes increased activity < 40%. It appears that guanylate cyclase activity in intact cells could be modulated by reversible modification of critical sulfhydryl groups, e.g., by thiol:disulfide exchange involving glutathione.

We reported before that analogues of GTP inhibited guanylate cyclase activity and now have found that several oxyanion compounds, such as vanadate, borate, molybdate, and tungstate, inhibited guanylate cyclase activity. 10 μM ortho-vanadate inhibited enzyme activity 90% with either Mn or Mg as the cation. The inhibition disappeared when vanadate was reduced in advance with DTT or ascorbate. Borate in mM concentration inhibited the activity in the presence of Mn ion but not Mg ion. The inhibition was not prevented by cis-diol compounds, such as fucose, mammose, ascorbate, or cyclobutanediol or mercaptoethanol. The inhibition by borate was uncompetitive (with MnGTP as substrate). Tungstate and molybdate both inhibited guanylate cyclase activity in the presence of Mn or Mg ion. The mechanism of inhibition is not known.

Activator of Guanylate Cyclase. Sometime ago we discovered that, after an early step in guanylate cyclase purification, enzyme activity was no longer proportional to concentration, i.e., the apparent specific activity increased with increasing enzyme concentration. From a fraction separated from the cyclase at this step, we have extensively purified a material termed activator that when added to assays restores proportionality between enzyme concentration and activity. Our continuing attempts to identify the activator are, however, still unsuccessful. Although it behaves in many ways as a large molecule, its stability to heat, acid, and alkali is most unusual for a protein. The purified activator survives treatment with trypsin or chymotrypsin but is apparently destroyed by pronase.

Preparation of Monoclonal Antibodies to Rat Liver Guanylate Cyclase. Serum from mice immunized with the highly purified enzyme contained antibodies that bound ^{125}I-labeled guanylate cyclase as demonstrated by radioautography of SDS gels of antigen-antibody complexes. Guanylate cyclase activity was precipitated from partially purified preparations by protein A when the immune serum was present. Spleen cells from immunized mice were fused with myeloma cells, and initial screening for hybrids producing antibodies to guanylate cyclase suggests that such are present.

Significance to Biomedical Research: Although at present information concerning the metabolism and functions of cyclic GMP is limited, it appears that this nucleotide may be of special importance in the development, physiology, and pathology of lung, vascular smooth muscle, and kidney.

Proposed Course: We shall further characterize the regulatory properties of purified guanylate cyclase. When monoclonal antibodies are prepared, they will be used in some of this work as well as for studies of factors that may control the amount of enzyme protein in cells. Attempts to identify activator and define its mechanism of action are continuing.

Publications:

Tsai, S.-C., Shindo, H., Manganiello, V.C., Adamik, R., and Vaughan, M.: Products of reaction catalyzed by purified rat liver guanylate cyclase determined by ^{31}P NMR spectroscopy. Proc. Natl. Acad. Sci. U.S.A. 77: 5734-5737, 1980.

Tsai, S.-C., Adamik, R., Manganiello, V.C., and Vaughan, M.: Reversible inactivation of soluble liver guanylate cyclase by disulfides. Biochem. Biophys. Res. Commun. 100: 637-643, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00614-04 CM

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Calmodulin-Dependent Cyclic Nucleotide Phosphodiesterase: Purification and Characterization

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

PI:	Randall L. Kincaid	Staff Fellow	CM	NHLBI
	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI
OTHERS:	Vincent C. Manganiello	Head, Section on Biochemical Physiology	CM	NHLBI
	Vsevoled Tkachuk	Guest Worker	CM	NHLBI

COOPERATING UNITS (if any)
Dr. Tkachuk was a Visiting Scientist under the US-USSR Exchange in the Cardiovascular Area, Problem Area 3, Myocardial Metabolism; Dr. James C. Osborne, Jr., MDB, NHLBI; Dr. Ellis S. Kempner, LPB, NIAMDD.

LAB/BRANCH
Cellular Metabolism

SECTION
Metabolic Regulation

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Calmodulin-dependent cyclic nucleotide phosphodiesterase has been purified to homogeneity from bovine and sheep brain using a rapid one-day method. The purified enzyme behaves as a dimer in the analytical ultracentrifuge. Fluorescence studies of the interaction between the purified enzyme and dansyl calmodulin indicate that formation of complex produces an increase in the affinity of calmodulin for Ca^{++}. Radiation inactivation studies, in addition to those with gel filtration and electrophoresis, suggest that enzyme monomer is fully active without calmodulin while dimer is inactive without calmodulin, thus providing a physical description for basal and calmodulin-dependent activity.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: To purify the calmodulin-dependent phosphodiesterase from brain and characterize the relationship between structure and enzyme activity. To characterize the interaction of calmodulin (and Ca^{++}) with the purified enzyme.

Methods Employed: An improved one-day method for purification based on Method I (described last year) has been developed which has been used to purify the enzyme from bovine and sheep brain to > 90% homogeneity. The 20,000 x *g* supernatant from a pH 6.0 extract of brain is applied to DEAE BioGel and eluted with a stepwise decrease in pH to 5.5. The eluate is concentrated and then applied to two successive calmodulin-Sepharose columns, the first one removing ∿ 20% of the enzyme and the major contaminants. The second affinity column is eluted directly onto an organomercurial affinity column and subsequently eluted with thiol.

A fluorescent derivative of calmodulin, dansyl calmodulin, has been prepared by incubation of calmodulin with dansyl chloride followed by exhaustive dialysis. The fluorescence spectrum (in polarized or unpolarized light) is then examined after addition of Ca^{++} and/or proteins that interact with calmodulin. In so doing, evidence for direct physical interactions can be obtained.

The method of radiation inactivation was used to estimate the functional size of the "basal" and "calmodulin-dependent" activity of the enzyme. Samples frozen at -150°C were irradiated with high energy electrons, and the loss of enzyme activity as a function of radiation dose was used to approximate the size of the active unit.

Major Findings: The use of the present purification method yields an enzyme from either bovine or sheep brain with a specific activity of 60-80 µmol cGMP hydrolyzed per min/mg protein and with an overall yield of 7-11%. A single peptide of 58,000 daltons (> 90%) is observed when analyzed by SDS gel electrophoresis and the protein appears homogenous in the analytical ultracentrifuge. (Its molecular weight based on sedimentation equilibrium is ∿ 128,000.) The enzyme activity is stimulated 6- to 10-fold by calmodulin with half-maximal stimulation at 5-7 x 10^{-10} M calmodulin, corresponding to a ratio of one calmodulin per mole of 60,000 dalton enzyme monomer. These data taken together with the sedimentation data suggest that purified phosphodiesterase is a dimer of identical subunits each capable of binding one molecule of calmodulin.

In collaboration with Drs. Tkachuk and Osborne, the interaction of the purified enzyme with dansylated calmodulin has been investigated. The fluorescence maximum of dansyl calmodulin (525 nm) is shifted to lower wavelengths (490 nm) upon interaction with Ca^{++} with an increase in fluorescence intensity. A further blue-shift and increase in intensity is observed upon addition of one of several calmodulin-binding proteins (Troponin I, modulator-binding protein, or phosphodiesterase). The reversal of the Ca^{++}-induced change in the fluorescence of the dansyl calmodulin requires considerably more EGTA when there has been formation of a phosphodiesterase-calmodulin complex than when

calmodulin alone is present. This indicates that the binding of phosphodiesterase increases the affinity of calmodulin for Ca^{++}. In addition, studies of fluorescence polarization with calmodulin and phosphodiesterase have been used to directly show the formation of a larger complex in the presence but not in the absence of Ca^{++}.

In an extension of previous findings, it was found that crude enzyme prepared immediately after homogenization behaved as a molecule of $M_r \sim 60,000$ on gel filtration but after 2 days its chromatographic behavior was that expected of a molecule with an M_r of 135,000. It was observed that calmodulin stimulation of activity in peak gel filtration fraction of the "aged" sample was about twice that observed when the fresh sample was used. This indicated a time-dependent formation of dimer enzyme with a concomitant increase in the dependence of activity on calmodulin.

Storage of a highly purified enzyme preparation at 4° (protein concentration 60 µg/ml) led to a rapid loss of calmodulin sensitivity without a great loss of total activity. Electrophoresis under nondenaturing conditions indicated that the enzyme underwent a change from predominantly oligomeric to monomeric species during this period. Analysis of SDS gels of the enzyme indicated no proteolytic degradation over this period, and the enzyme could still be quantitatively bound to and eluted from calmodulin-Sepharose. These data suggested that the loss of calmodulin responsiveness was related to the appearance of a monomeric species, which can interact with but is not further stimulated by calmodulin.

In collaboration with Drs. Kempner and Osborne, radiation inactivation studies relating the subunit structure to enzyme activity were carried out. Low doses of radiation caused an increase in the "basal" activity which with higher doses decayed with a slope corresponding to a target of $\sim$ 60,000 daltons. By contrast, the calmodulin-dependent activity followed a single exponential decay with a slope of $\sim$ 105,000 daltons. The degree of activatability also decayed exponentially as a function of dose. These data suggest that dimers, which are inactive in the absence of Ca^{++} and calmodulin, are destroyed by increasing radiation, giving rise to additional active monomers (i.e., "basal" activity). This progressively decreases the stimulation of activity when assayed in the presence of calmodulin. These interpretations were consistent with results predicted by computer-assisted simulation of the proposed model. This monomer-dimer hypothesis provides for the first time an explanation in molecular terms for the "basal" and "calmodulin-dependent" activities of the enzyme.

Significance to Biomedical Research: The effects of many biochemical regulators, such as hormones, on mammalian cells are mediated by altering the rates of synthesis and/or degradation of cyclic nucleotides. The understanding of the properties of the enzyme regulating cyclic nucleotide degradation is important for the understanding of normal and pathological cellular activity and may permit design of rational therapeutic approaches. The well-established significance of calmodulin in control of diverse cellular functions makes the elucidation of its mechanism of action important for the understanding of Ca^{++}-regulated events.

Proposed Course: With our improved method of purification, we are now able to rapidly prepare amounts of the calmodulin-activated phosphodiesterase that will enable us to proceed with studies of the structure of the protein as well as further investigation of physical properties of the enzyme and the mechanism of its activation by calmodulin.

Publications:

Kincaid, R.L., Manganiello, V.C., and Vaughan, M.: Calmodulin-activated cyclic nucleotide phosphodiesterase from brain: Changes in molecular size assessed by gel filtration and electrophoresis. J. Biol. Chem., in press, 1981.

Kincaid, R.L., Kempner, E., Manganiello, V.C., Osborne, J.C., Jr., and Vaughan, M.: Calmodulin-activated cyclic nucleotide phosphodiesterase from brain: Relationship of subunit structure to activity assessed by radiation inactivation. J. Biol. Chem., in press, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00615-04 CM

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Production and Characterization of Monoclonal Antibodies against Bovine Brain cGMP Phosphodiesterase

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

PI:	Mary Ann Danello	Staff Fellow	CM	NHLBI
	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI
OTHERS:	Randall L. Kincaid	Staff Fellow	CM	NHLBI
	Vincent C. Manganiello	Head, Section on Biochemical Physiology	CM	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Cellular Metabolism

SECTION
Metabolic Regulation

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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)

Recent advances in the production of monoclonal antibodies, using specialized cell fusion techniques, permit the isolation and characterization of extremely specific antibodies to complex antigens. Such antibodies made in response to highly purified calmodulin-activated cGMP phosphodiesterase from brain should be useful for probing functional domains in the enzyme as well as for studies of enzyme localization and regulation at the cellular level.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: The objective of this work is to isolate and characterize monoclonal antibodies to a highly purified preparation of calmodulin-activated cGMP phosphodiesterase from bovine brain. Because of their extreme specificities, such antibodies will recognize individual antigenic sites on the phosphodiesterase molecule and can be utilized to study the interaction of the enzyme with other proteins which modulate its activity, to establish the cellular localization and distribution of the enzyme, and to compare the antigenic properties of different phosphodiesterases from different tissues and species.

Methods Employed: Two methods of raising monoclonal antibodies were used. The first, employing an *in vitro* immunization procedure, was described by Luben and Mohler (1980). The cells from one Balb C mouse spleen were cultured in the presence of the highly purified phosphodiesterase and other growth factors for four days before fusion with NS-1 myeloma cells according to the methods of Kohler and Milstein (1975). The more successful procedure involved fusing NS-1 myeloma cells with spleen cells from an immunized Balb C mouse whose serum contained antibodies against the enzyme.

For detection of antibodies, polyvinyl, V-shaped, 96-well plates were coated with purified rabbit anti-mouse IgG. Hybridoma supernatants were then added and, after incubation to permit binding of IgG or IgM, the wells were washed. In the final step, ^{125}I-phosphodiesterase which should bind to wells containing specific antibody was added.

Highly purified calmodulin-activated cGMP phosphodiesterase from bovine brain was prepared by procedures developed in this laboratory.

Major Findings: The serum from Balb C mice repeatedly injected with highly purified native cGMP phosphodiesterase and with chymotryptic fragments of the enzyme specifically bound ^{125}I-phosphodiesterase (10-fold above blank) in the solid phase radioassay but did not significantly inhibit the activity of the calmodulin-activated enzyme when assayed in solution. Antibodies in the mouse sera were also capable of binding specific fragments of the phosphodiesterase generated by limited digestion with α-chymotrypsin. Spleen cells from mice with serum antibodies to the phosphodiesterase were successfully fused with myeloma cells. Hybrid cells producing either IgM or IgG antibodies were detected but have not yet been successfully cloned and maintained.

In an attempt to increase the sensitivity of the detection system for monoclonal antibodies and to avoid some of the variability that appears to be related to components of the medium in which the hybrid cells are grown, we have recently modified the assay. The test wells are first coated with purified phosphodiesterase, the medium samples to be tested for the presence of antibodies are then introduced, and, finally, any antibody that has bound is detected by its ability to bind ^{125}I-labeled sheep anti-mouse $F(ab')_2$ fragments. This assay solved some of the problems encountered with the procedure used earlier and, in addition, saves time, which is an important consideration with the large numbers of samples that must be assayed.

To provide an antiserum to the phosphodiesterase in amounts sufficient to carry out preliminary immunological studies before monoclonal antibodies are available, we are immunizing a goat. Because of our improved procedure for purification of the enzyme (Report No. Z01 HL 00614-04 CM), we now can relatively easily prepare the amounts of phosphodiesterase required for this.

Significance to Biomedical Research: Production of monoclonal antibodies to an important enzyme of cyclic nucleotide metabolism is important not only for understanding the nature of the enzyme itself but also for eludicating the processes which regulate its activity in mammalian cells.

Proposed Course: Screening of hybrids from the most recent fusion for antibody production is proceeding. Antibodies will be used to probe functional domains of the enzyme, to look for possible relationships between this and other phosphodiesterases, and for localization and quantification of the enzyme protein in cells.

Publication: Vaughan, M., Danello, M.A., Manganiello, V.C., and Strewler, G.J.: Regulation of Cyclic Nucleotide Phosphodiesterase Activity. In Dumont, J.E., Greegard, P., and Robison, G.A. (Eds.): Advances in Cyclic Nucleotide Research. New York, Raven Press, 1981, Vol. 14, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00617-05 CM

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
The Mechanism and Action of Anti-Inflammatory Drugs: Effects on Neutrophil Mobilization in Carrageenan- and Dextran-Induced Inflammation

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

PI:	Theresa N. Lo	Research Chemist	CM	NHLBI
OTHER:	Michael A. Beaven	Head, Section on Cellular Pharmacology	CM	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Cellular Metabolism

SECTION
Cellular Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.1	PROFESSIONAL: 1.1	OTHER: 0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Carrageenan injected into the rat pleural cavity induces after a short delay extravasation of plasma protein, the appearance of a neutrophil chemotactic activity (CA) and, by 4 hr, the infiltration of large numbers of neutrophils (80-140 x 10^6 cells/cavity). Indomethacin (I) given intravenously suppressed these reactions primarily by suppressing protein exudation; both total protein and CA were reduced equally but the specific activity (CA/mg protein) was unchanged. In contrast to carrageenan, the Dextrans (T10, 40, 70, 150, 500, 2000) provoked upon injection mast cell degranulation and rapid accumulation of watery exudate with little protein and a few leukocytes. This reaction was not suppressed by I. The studies indicated that extravasation of water and protein may occur by different mechanisms and that, in the examples studied, leucocytaxia was associated with protein leakage.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: Our earlier work on the mediators involved in inflammatory reactions and the present studies indicate two types of inflammatory responses: 1) one characterized by mast cell degranulation and rapid fluid accumulation and 2) inflammation of slow onset in which mast cells remain intact and in which there is progressive accumulation of fluid, plasma protein, and neutrophils over the course of 2-8 hr. The first type is observed after scalding or the injection of dextran, the second following injection of carrageenan and other high molecular weight substances. Carrageenan is not in itself chemotactic for neutrophils but is believed to activate endogenous precursors of chemotactic activity. Although there is evidence that activation of the complement system mediates the recruitment of neutrophils in response to a variety of high molecular weight substances (e.g., zymosan, bacterial lipopolysaccharides), little data are available on the mode of action of carrageenan.

This study was designed to assess the relationship of plasma protein exudation, generation of chemotactic activity, and neutrophil accumulation in response to carrageenan and the effects of indomethacin treatment on these parameters. For comparison, similar studies were undertaken with dextrans which induce the first type of inflammatory response. The data suggest that the generation of chemotactic activity is dependent on availability of plasma protein and that indomethacin's primary action may be suppression of exudation of plasma protein. A serendipitous finding was that a dextran from one manufacturer provoked both types of reaction, fluid accumulation of rapid onset followed by a slower accumulation of protein and neutrophils.

Methods Employed: Purification of Neutrophils from Rat Blood. Samples of heparinized rat blood were obtained by cardiac puncture. Neutrophils were purified by the procedure of Boyum (Scand J. Clin Lab. Invest. 21: 77, 1968). Preliminary studies (n = 13) indicated that preparations containing 42% neutrophils could be obtained from normal rats (neutrophils ~ 12% of white cells in blood) compared to 92% neutrophils in preparations from human blood, in which 57% of white cells are neutrophils. Since the percentage of circulating neutrophils increases to over 50% 3 hr after the intrapleural injection of 0.5 mg carrageenan in rats (Almeida *et al*., J. Pharmacol. Exp. Ther. 214: 74, 1980), this strategy was employed to obtain blood with an increased neutrophil count. Blood obtained from 3 such rats yielded ~ 1.1 x 10^6 leukocytes (> 90% variable) of which 75-96% (mean 82%) were neutrophils.

Preparation of Neutrophils from Pleural Exudate for Assay of Chemotactic Activity. Carrageenan was injected intrapleurally into 3 rats and the pleural fluid collected 3 hr later. The exudate and washings were pooled. The cells were washed three times and suspended in a Gey's-HEPES (25 mM), BSA (2% w/v) solution (2.5 x 10^6 cells/ml) to use in assays of chemotactic activity. More than 90% of the cells were neutrophils and viability exceeded 95%.

Collection of Cell-Free Pleural Exudate for Assay of Histamine, Protein and Chemotactic Activity. Rats were given saline or indomethacin (5 mg/kg) intravenously and 30 min later, under light ether anesthesia, carrageenan (500 µg in 0.1 ml), Compound 48/80 (0.05 mg in 0.1 ml), or dextran (60 mg in 1 ml or as indicated) was injected into the pleural cavity. Rats were killed

by ether, the exudate was aspirated into a plastic syringe, and the cavity was washed with 1 ml of the Gey's-HEPES (25 mM). The exudate and washings were pooled. The volume reported was corrected by subtracting the 1-ml wash. Total and differential cell counts were determined by standard techniques described in our previous publication. The remainder of the exudate was centrifuged at 600 x g for 10 min at 4°C. Samples of the supernatant fluid were assayed for protein by the method of Lowry, for histamine by a radioenzymatic assay, and for chemotactic activity as described below.

Assay of Chemotactic Activity. A modified Boyden Chamber with a micropore filter (3-μm diameter pores) was used. The upper compartment contained 0.2 ml of cell suspension (500,000 cells). The lower compartment contained 1.2 ml of one of the following in Gey's-HEPES medium: (1) the cell-free fraction of the pleural exudate diluted in Gey's-HEPES to give the indicated protein concentration; (2) 10 mM F-Met-Leu-Phe (to test cell responsiveness); (3) carrageenan as indicated; or (4) Gey's-HEPES medium alone. The chambers were placed for 90 min (37°C) in an atmosphere of 95% air-5% CO_2, 100% humidity, after which the filter was rinsed, fixed in formaldehyde solution, and stained with hematoxylin. All samples were run in duplicate.

Calculation of Chemotactic Activity. Migration of neutrophils was assessed by the "microscopic sectioning" technique of Maderazo and Woronick (Clin. Immunol. Immunopathol. 11: 196, 1978). The cells were counted at 10 μm (X 40 objective) intervals. Three or more randomly selected fields per interval were examined and the average cell number (B) per field calculated. The locomotion index (LI) was calculated from the formula: $LI = \frac{\Sigma (A \times B)}{\Sigma B}$ where A is the distance migrated from origin in μm (i.e., 1, 2, 3, etc., x 10 μm).

Major Findings: A. Studies with Carrageenan. Evidence for the presence of chemotactic activity in cell-free pleural exudates obtained after injection of carrageenan. The presence of chemotactic activity was evident from the enhanced migration of neutrophils (obtained from peripheral blood or carrageenan exudates) in response to small amounts of cell-free pleural exudates. Typical values (mean LI ± SE, n = 6) obtained for cell migration were: 21.8 ± 0.4 for medium alone; 45.3 ± 1.2 for 10^{-8} M F-Met-Leu-Phe; 27.7 ± 1.8, 30.9 ± 0.8, and 37.7 ± 1.2 for samples of exudate containing 0.75, 1.0, and 1.5 mg exudate protein, respectively. In all cases, the migration profiles of neutrophils in the presence of exudate protein were clearly separated from those observed with medium alone. Additional studies gave no indication that carrageenan was a chemotactic stimulant for either blood or exudate neutrophils.

The relationship between chemotactic activity and protein content of exudate. A chemotactic response was generally not detectable with amounts of exudate protein less than 0.5 mg protein (7 experiments). Linear increases in chemotactic response were observed between 0.5 and 1.5 mg exudate protein (2 experiments). Based on these results, measurement of chemotactic activity was assessed with 3 concentrations of exudate protein, 0.75, 1.0, and 1.5 mg protein (which fall within the linear portion of the curve) and in each experiment exudates were collected from matched pairs (2 control and 2 drug-treated rats)

of rats. A total of 4 experiments were conducted in this manner and the data pooled.

Effect of indomethacin on generation of neutrophil chemotactic factor(s). The values for chemotactic activity (LI/mg or exudate protein) for both control and drug-treated rats were similar when the values for LI were calculated from the regression lines for each exudate (6.2 ± 0.9/mg protein for control versus 6.8 ± 0.7/mg protein for indomethacin-treated rats, n = 10). Indomethacin treatment (5 mg/kg i.v.) reduced the accumulation of cells (-41%), volume of exudate (-47%), total protein (-56%), and total chemotactic activity (-58%) to similar degrees. Thus, while the specific chemotactic activity (i.e., LI/mg protein) was unchanged, total chemotactic activity generated was reduced in proportion to protein content.

B. Studies with Dextrans and other Inflammatory Agents. Exudate composition 30 min after the injection of inflammatory agents. The dextrans tested included T10, T40, T70, T150, T500, T2000 (Pharmacia), and T250 (Sigma Chemical Company). The last was found later to be contaminated with trichloroacetic acid-soluble peptide material. Accumulation of fluid (0.9-1.2 ml) was evident 30 min after the injection of all the dextrans tested and Compound 48/80 but little after the injection of carrageenan or saline (< 0.2 ml). The number of white cells (4-8 x 10^6 cells) and protein (2-8 mg) recovered from the pleural cavity after all treatments was similar to that obtained by washing the pleural cavity of untreated rats. An exception was after treatment with Compound 48/80 where protein increased to 21 mg.

Though the histamine content of the cavity fluid and washings was high (between 9 and 19 µg), less than 1% of this histamine was recovered in the extracellular fluid of rats treated with saline or carrageenan. Extracellular levels were increased significantly (0.04 to 0.26 µg/ml) after the injection of dextrans and Compound 48/80, and, in the case of Compound 48/80-treated rats, the cellular histamine content was decreased by 80%. This decrease suggested that histamine release was more extensive with Compound 48/80. This was confirmed by morphological examination of the exudates. All mast cells remained intact in rats treated with either NaCl or carrageenan. After T10, 35% of the mast cells showed signs of partial degranulation and, after T2000, 58% of the mast cells were degranulated to varying extents. Few intact mast cells (< 2%) were observed in exudates of 48/80-treated rats. Thus, mast cell degranulation was observed only with agents that provoked an immediate accumulation of fluid.

Exudate compositions 4 hr after the injection in inflammatory agents and the effect of indomethacin. With all agents, exudate volumes ranged from 0.9 to 1.8 ml, but, with the exceptions noted below, the exudates contained little protein (< 9 mg) and few cells (< 12 x 10^6). Only two agents, carrageenan and Dextran T250, produced a large accumulation of neutrophils (80-120 x 10^6 cells) and protein (42-54 mg protein) in the pleural cavity. After carrageenan, the protein content of the exudate (49 mg/ml) was almost as high as that (60 mg/ml) in plasma. Small increases (19 x 10^6 cell and 19 mg protein) were observed after the injection of the highest molecular weight of dextran tested (T2000, Pharmacia).

In all experiments, protein content and cell count were highly correlated ($p < 0.001$ R 0.95) but neither of these correlated with fluid volume.

Indomethacin (5 mg/kg i.v.) given 30 min before the intrapleural injection of drugs was found to be effective only in suppressing the response to carrageenan and, to a lesser extent, T250. The responses to other dextrans were unaffected.

Significance to Biomedical Research and the Program of the Institute: Carrageenan- and dextran-induced edema appear to be useful models to study the effects of drugs on different types of inflammatory reactions. With these models, we have shown that 1) mast cell degranulation (and early edema formation) and neutrophil accumulation are unrelated phenomena, although both may occur in response to a single agent, 2) increased vascular permeability to proteins appears to be associated with production of chemotactic factor(s) and neutrophil accumulation, 3) indomethacin and other nonsteroidal anti-inflammatory drugs inhibit protein exudation and the recruitment of white cells but not the edema induced by mast cell degranulation. The production of an exudate almost devoid of protein, on the one hand, and of exudate with a protein composition almost identical to that of plasma, on the other, suggests that our understanding of the mechanisms responsible for increased vascular permeability is incomplete and cannot be explained by a single process (opening of capillary pores, for example). The data provide an explanation as to why anti-inflammatory drugs are effective in some forms of inflammation and not in others.

Proposed Course: The contribution of histamine release (and other mast cell constituents) to early edema (fluid) response and complement activation to neutrophil accumulation will be assessed in studies with antihistamine drugs and antibodies to components of the complement system.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00619-05 CM

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Role of Histamine in Gastric Secretion. Studies with Isolated Gastric Mucosal Cells

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

PI:	Michael A. Beaven	Head, Section on Cellular Pharmacology	CM	NHLBI

COOPERATING UNITS (if any)
Drs. Andrew H. Soll, Gordon Kauffman, and Morton I. Grossman, Center for Ulcer Research and Education, Wadsworth V.A. Hospital, Los Angeles, California

LAB/BRANCH
Cellular Metabolism

SECTION
Cellular Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.2	0.1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Studies with highly purified preparations of cells from collagenase digests of gastric mucosa suggest that in some species histamine (and histidine decarboxylase) is located in mast cells which lay in close proximity to the parietal cell. In rodents, these substances are present in enterochromaffin-like (ECL) cells which are embedded in the pit of the gastric gland. A mutant mouse (WB/ReN-W^v) which has few mast cells was found to have near normal histamine levels in brain and stomach and markedly deficient levels (2-8% of normal) in other tissues when compared to congeneric normal mice. Studies of the clearance of histamine across the vascular beds of stomach and other organs during i.v. perfusion of histamine in dogs indicated almost complete removal of circulating histamine through the hind limb, partial clearance by visceral organs and a half-life for histamine in the whole animal of 25 sec. Plasma levels of less than 4×10^{-7} M histamine appear to be sufficient to induce gastric secretion at 25-50% of the maximal rate.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: The finding that the histamine H_2 receptor antagonists block gastric secretion in a variety of species provided the first direct evidence that histamine has an essential role in gastric secretion. Histamine's relationship to other mediators of gastric secretion, gastrin and acetycholine, is still, however, poorly understood. Nor is it known how histamine is released or how it reaches the acid-secreting parietal cell. The successful separation of different cell populations in dog gastric mucosa by Soll and Grossman at the Center for Ulcer Research and Education (CURE), Wadsworth V. A. Hospital, Los Angeles, and their subsequent collaboration with us has given us an opportunity to design experiments to answer some of these questions. This approach led to the identification of the "histamine-containing cell" in the gastric mucosa of dog and rat. In this report, we describe studies on 1) the preliminary separation of histamine-containing cells from human gastric mucosa; 2) the distribution of histamine in the gastrointestinal tract of a mutant strain of mouse that has markedly reduced (98%) numbers of mast cells; 3) the clearance of histamine across the gastric vascular bed of dog during i.v. perfusion of histamine in concentrations that elicit 25, 50, and 75% of the maximal rate of gastric secretion; and 4) isolated canine gastric mast cells in short-term culture. The latter studies, still unsuccessful, were designed to test putative mediators of histamine release. The mechanism of histamine release remains the unidentified link in the sequence of events leading to gastric secretion. The studies were performed both at the NIH and during a visit by the principal investigator to Wadsworth, funded in part by the NHLBI and UCLA.

Methods Employed: Tissues were obtained as described in previous reports. "Normal" human mucosa was obtained from specimens obtained after partial gastrectomy. Gastric mucosa and other tissues were treated sequentially with a collagenase preparation, EDTA, and then a second time with collagenase. The cells were harvested from the digested suspension and then separated in a Sorvall zonal rotor through an albumin density gradient. This procedure separated the cells into histamine, parietal, and chief cell-enriched fractions. Each of these was further fractionated according to cell size with the Beckman elutriator. Rat peritoneal mast cells were fractionated by elutriation. At all stages, cell viability was monitored by microscopy and dye exclusion tests. The fractions were divided into portions of about 10^6 cells, and duplicate sets were shipped from CURE to the NIH.

At the NIH, the fractions were assayed for DNA, histamine, and serotonin content as well as for histidine decarboxylase, DOPA decarboxylase, histamine-N-methyltransferase (HNMT) and diamine oxidase activities, using the microassays developed in this laboratory. These assays have been described in previous annual reports. Assays were also performed by the principal investigator on fresh samples at CURE.

Rat peritoneal mast cells and dog gastric mucosa mast cells were dispensed into coated (gelatin, fibrin, polylysine) plates and incubated in media supplemented with various peptide hormones and antibiotics. The cells were harvested 48 and 72 hr later. Sterile equipment was used for these experiments (these studies were performed at CURE by Dr. Soll).

Major Findings: Isolation and Properties of the Histamine-Containing Cells in Dog Liver and Dog and Human Fundic Mucosa. Profiles obtained by separation of different cell fractions produced a single sharp peak of histamine for both liver and mucosa. In all tissues, the highest histamine concentration was found in fractions containing cells with a mean density of 1.080 and a diameter of 11 μm. Mast cells constituted more than 80% of the cells in these fractions. The average histamine content was 2.5 ± 0.3 pg/cell (n = 3) and 1.9 (n = 1) for dog and human mucosa and 2.1 ± 0.1 (n = 3) pg/cell for dog liver. Electron micrographs confirmed that a high proportion of the cells possessed the typical morphology of mast cells with characteristic dense granular structure.

Measurement of DNA as well as histamine at all stages of purification indicated that at least 70% of the histamine content of the unfractionated dog mucosal cells was present in mast cells. It was calculated that the mucosa contained 3×10^7 mast cells/g (about one mast cell per 3 parietal cells) and that liver contained 1×10^7 mast cells/g tissue.

In dog, histidine decarboxylase activity was located exclusively in the mast cell fractions of the mucosa and liver cell preparations. In the mucosal cells, the activity was 25% of that in the liver and 20% of that in the rat ECL cell. The mast cells were devoid of histamine methyltransferase activity. As reported last year, most of this activity in dog mucosa was associated with the parietal cell.

Clearance of Histamine Across Various Vascular Beds in Dog. Catheters were distributed into various regions of the vascular system of anesthetized dogs. Histamine was perfused into the femoral vein at the rate of 20, 70, or 160 ng histamine/min/kg, which has been shown to induce gastric acid secretion at 25, 50, and 75% of the maximum flow rate, respectively. At a time when plasma histamine levels had equilibrated (15 min), blood samples were withdrawn into heparinized tubes and processed to collect plasma (experiments performed by Dr. Kauffman). With the two slower rates of perfusion, plasma histamine levels in the right atrium increased by less than 5 ng/ml (normal histamine level, 1.5 ng/ml). With the highest rate of perfusion, the levels increased to 35 ng/ml in the right atrium and 41 ng/ml in the carotid artery. Significant variations in the plasma histamine levels were noted, however, in different regions of the venous system. In vessels draining the hind limbs, histamine levels were 0.2 ng/ml (normal < 0.2 ng/ml), an indication of almost complete removal of free histamine. Clearance of similar magnitude (> 90%) was observed across the kidney. In the mesenteric, gastric, and portal veins, the plasma levels were 9, 12, and 17 ng/ml, respectively (normal 0.2-0.6 ng/ml), and in the hepatic vein, 6 ng/ml (normal 0.7 ng/ml). These data suggest that some histamine had escaped degradation, possibly by passage through vascular shunts. Upon cessation of infusion, histamine levels in the right atrium decayed rapidly with a half-life of about 25 sec. Thus, substantial gastric secretion can be induced with circulating histamine levels of less than 0.4 μM and near maximal secretion with levels of approximately 3 μM. As in other species, histamine is rapidly inactivated in the circulation.

Studies with Mast Cell Deficient Mutant Mice (WB/ReN-W^v). These were obtained from Dr. Hansen (Division of Research Services) along with their

litter mates (+/+). It has been reported by Kitamura et al. (Blood 52: 447, 1978) that a similar line of mice contains no detectable mast cells in tissues except skin, where the number of mast cells are less than 2% of that in the congeneric +/+ mice. Animals were killed at different ages and tissues removed for the assay of histamine. Histamine levels in adult w/w^{α} mice in tissues, such as blood, liver, kidney, spleen, lung, heart, and skin, were 2-8% of those in the same tissues from congeneric (+/+) normal mice. Only in brain, stomach, and duodenum were histamine levels comparable to those in +/+ mice. The presence of histamine in stomach is consistent with the report of Hakanson and his associates (see Gastroenterology 77: 800, 1979) that mouse, like rat, stomach contains no mast cells but abundant numbers of ECL cells.

Studies of Histamine Release from Histamine-Containing Cells Grown in Short-Term Culture. Rat peritoneal mast cells were fully responsive to the histamine-releasing action of Compound 48/80, Na^+ ionophore, and other histamine-releasing agents. Mast cells from dog gastric mucosa showed no response (histamine release) to these agents, ascaris antigen and various agonists and hormones (gastrin, carbachol, somatostatin, prostaglandins, and prostacyclin) and a partial response to Na^+ ionophore. Despite these negative results, this work is still being pursued for the reasons outlined in the objectives.

Conclusions and Significance to Biomedical Research: The studies provide some indication of the levels of histamine required to activate parietal cells in dog and reveal differences in the location of histamine in gastric glands from different species. In dog, the mast cells abut onto the parietal cell, and the presence of high histamine methyltransferase activity in the parietal cell probably ensures that little free histamine escapes into the gastric circulation in this species. In man, like the dog, the gastric histamine stores are located in mast cells within the gastric gland. In rat (last year's report) and possibly mouse (this report), gastric histamine is sequestered in an APUD-type cell now designated an enterochromaffin-like (ECL) cell. In these two species, the ECL cells are embedded in the base of the gastric pits some distance from the parietal cell. Presumably, histamine must diffuse across extracellular spaces or into the small blood vessels of the lamina propria before reaching the parietal cell. Unlike other species, increases in urinary histamine levels are observed in rat during periods of gastric stimulation. Although the present studies begin to provide a picture of histamine's location and, upon release, its disposition, we are still uncertain as to the mechanism(s) of its release.

Proposed Course: We will continue to focus our studies on possible mechanisms of histamine release from histamine-containing cells by use of short-term culture experiments. More detailed biochemical studies (e.g., activation of adenylate cyclase, Ca^{++} flux) will follow once the agents inducing histamine release have been identified.

Publications: Soll, A.H., Lewin, K.J., and Beaven, M.A.: Isolation of histamine containing cells from rat gastric mucosa: Biochemical and morphological differences from mast cells. Gastroenterology 80: 717-727, 1981.

Beaven, M.A., Soll, A.H., and Lewin, K.J.: Biochemical characterization of mast cells from canine fundic mucosa and liver. Gastroenterology, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00620-04 CM

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Regulation of Histamine Synthesis and Release in Tissues

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

PI:	Michael A. Beaven	Head, Section on Cellular Pharmacology	CM	NHLBI
OTHER:	Barbara M. Bayer	Staff Fellow	CM	NHLBI

COOPERATING UNITS (if any)

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.9	0.3	0.6

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Histamine synthesis by purified intact rat peritoneal mast cells, as measured by formation of β-^{3}H-L-histidine or release of $^{14}CO_2$ from ^{14}C-carboxyl-labeled histidine, was 10 to 30 times greater than that of disrupted cells or soluble extracts of these cells. Loss of activity was evident whether cells were disrupted by sonification, freezing and thawing, or lysis both in the absence and presence of agents known to preserve enzyme activity. Studies with decarboxylase inhibitors indicated that a specific histidine decarboxylase was responsible for histamine formation in both the intact cells and cell extracts. In the presence of subsaturating concentrations of histidine, various histidine analogs and glutamine inhibited histidine uptake and histamine formation in intact mast cells but did not inhibit synthesisk in cell extracts. These data indicate that, at physiological concentrations of histidine, blockade of histidine transport may limit histamine synthesis in the intact cell and that measurement of histidine decarboxylase activity in tissue homogenates or cell extracts may not reflect the rate of histamine synthesis *in vivo*.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: Histamine is found in mammalian tissues in amounts ranging from less than 0.1 μg/g in blood to over 50 μg/g in gastric mucosa and lung and 1 mg/kg in mastocytomas. The enzyme responsible for histamine synthesis, histidine decarboxylase (EC 4.1.1.22), has been identified in extracts of many tissues. This enzyme is specific for histidine and is inhibited by α-methylhistidine but not, except in high doses, by α-methylDOPA. These characteristics distinguish it from the nonspecific aromatic amino acid decrboxylase (DOPA decarboxylase, EC 4.1.1.26), which has a low affinity ($K_m = 10^{-2}$ M) for histidine and is inhibited by α-methyl-DOPA but not by α-methylhistidine. Paradoxically, histidine decarboxylase activity in extracts of most tissues is low and is insufficient to account for the high histamine levels, unless histamine turnover occurs at an extremely low rate.

Last year, we presented preliminary evidence that histamine synthetic activity of intact rat peritoneal mast cell is largely destroyed upon cell disruption. In the present studies, the phenomenon is examined in more detail in a variety of histamine-containing cells. The relationship between histidine uptake and histamine synthesis in these cells is also examined.

Methods Employed: Collection and Purification of Cells. Cells were prepared by elutriation or Ficoll density gradient separation. Male, Sprague-Dawley rats (180 to 300 g) were killed by decapitation, and 10 ml of Hanks' balanced salt solution was injected into the abdominal cavity. The abdomen was then massaged for 90 sec, the abdominal wall was opened, and the fluid removed by syringe. The peritoneal cells were washed and resuspended in 10 ml of the Hanks' solution.

In studies of the distribution of histamine and histidine decarboxylase in the different cell fractions, the cell suspension (∿ 150 x 10^6 cells) was loaded into an elutriator rotor (Beckman Instruments, Spinco Division) and separated into 10 fractions by successive decreases in rotor speed. The final fraction (#10) consisted of the residual material remaining in the separation chamber. Cell counts and size analysis were performed using a Particle Data Counter. The viability of cells was assessed by trypan blue exclusion, light and electron microscopy, as well as by their ability to degranulate in response to Compound 48/80.

The effects of various agents and treatments in histidine decarboxylase activity and histidine transport was studied with mast cells purified from peritoneal suspensions (∿ 20 x 10^6 cells) by a single step Ficoll gradient procedure. Purified mast cells were dispersed in 10 ml of Hanks'-HEPES-albumin solution and then sedimented by centrifugation at 200 x g for 10 min. The yield was 4-6 x 10^6 cells (87 ± 2 mast cells, mean ± SE, n = 20).

Other preparations studied included both rat peritoneal and pleural cell suspensions, partially purified fraction of rat stomach enterochromaffin-like (ECL) cells, purified mast cell suspensions from dog liver and gastric mucosa. and a soluble extract of rat gastric mucosa (soluble histidine decarboxylase). The ECL cells and mast cells from dog were purified by procedures developed by Soll et al. (see project Z01 HL 00619-05 CM for details).

Experimental and Assay Procedures. For the assay of histamine and enzyme activities, samples (1 ml) of cell suspensions were centrifuged and the supernatant

fluid aspirated. Cells were dispersed in Hanks' salt solution containing 10 mM HEPES buffer, pH 7.4 (0.5×10^6 cells/200 µl), or other media as indicated in the text. Where noted, cells were disrupted by sonification (Kontes Ultrasonicator, maximum setting) for 10 sec, freezing and thawing three times (on Dry Ice), or by lysing with water or Triton X-100, 4% w/v. Where indicated, protease inhibitors were added to the medium before cell disruption. Histidine and DOPA decarboxylase activities were assayed in intact and disrupted cells by measurement of $^{14}CO_2$ release from ^{14}C-carboxyl-labeled L-amino acids. The specificity of the assay for histidine decarboxylase activity was verified by incubating the samples with β-^{3}H-(side chain carbon)-L-histidine (20 nCi) in addition to the ^{14}C-carboxyl-labeled L-histidine (20 nCi); the composition of incubation mixture was otherwise identical to that used in the $^{14}CO_2$ release assay. After trapping the $^{14}CO_2$ in Hyamine, the reaction mixture was assayed for ^{3}H-histamine by isotope dilution derivative analysis. Histamine and serotonin were assayed by the standard enzymatic isotopic assays developed in this laboratory.

Uptake of β-^{3}H-L-histidine was measured by incubation of 10^5 mast cells with 100 nCi (20 pmol) β-^{3}H-L-histidine in 100 µl Hanks'-HEPES-albumin solution for 2.5 min (or as indicated) at 37°C or 0°C. Controls (blanks) contained, in addition, 20 mM unlabeled histidine. The cells were deposited by centrifuging (8000 x g) the samples in a Microfuge tube through 250 µl fetal calf serum for 1 min. The pellets were recovered for radioassay. All determinations were done in triplicate (3 test and 3 blanks), and the difference in ^{3}H content of the test and blank pellets was designated as "uptake."

Major Findings: 1) Histamine Synthesis in Intact and Disrupted Peritoneal Mast Cells. Separation of rat peritoneal cells by elutriation yielded a single peak of cells containing histamine. Mast cells contributed 80 to 85% of the cells in the fractions with maximal histamine content of 16 ± 1.8 pg and serotonin content of 0.5 ± 0.1 pg/mast cell (n = 5, all values mean ± SE). The distribution of histidine decarboxylase activity paralleled that of histamine, although the rate of histamine formation by intact cells was 20 times greater than that of soluble extracts of these cells. The magnitude of this difference was similar when histidine decarboxylase activity was measured by $^{14}CO_2$ release from ^{14}C-carboxyl-labeled histamine or by the formation of ^{3}H-histamine from side chain-labeled β-^{3}H-histidine. The decarboxylation of histidine in both the intact cells and the extracts was attributable to specific histidine decarboxylase in that this activity was inhibited by α-methylhistidine but not, except in high concentrations, by α-methylDOPA.

Loss of activity was evident when mast cells were disrupted by freezing and thawing, lysing with water or detergent, when protease inhibitors (soybean trypsin inhibitor; dimethyl sulfonyl fluoride, 100 µg/ml; or mixture of antipain, leupeptin, chymostatin, 20 µg/ml), substrates (L-histidine and pyridoxal phosphate), or preservatives (polyethylene glycol 400 and Cleland's reagent) were present, or in the presence or absence of sodium. There was no loss of activity with the soluble gastric enzyme preparation with any of the above treatments or when the gastric preparation was mixed with disrupted cell suspensions (34 ± 0.3 versus 42 ± 0.3 pmol/hr/mg tissue).

In contrast to the above, preliminary studies (4 experiments) have indicated no loss of histamine synthetic activity upon mast cell degranulation with Compound 48/80. This finding will be examined further (see Proposed Course).

2) Changes in Kinetic Parameters upon Disruption. The rate of histamine synthesis was proportional to the amount of sample in amounts up to 0.5 x 10^5 cells/20 µl for intact cells or 4 x 10^5 cells/20 µl for cell extracts. The relationship of substrate concentration and histamine synthetic activity, as indicated by Lineweaver-Burk plots, showed that upon disruption of cells V_{max} decreased by more than 90% (790 ± 220 versus 21 ± 8 pmol/hr/10^6 cells, mean ± SE for 3 experiments) and the apparent K_m approximately doubled (50 ± 10 µM versus 110 ± 40 µM). The apparent K_m for the soluble cell extract was not significantly different from that of soluble extract of gastric mucosa (45 ± 15 µM for 3 preparations). Histamine synthesis by both the soluble mast cell and gastric enzyme but not the intact cells was partially dependent on pyridoxal phosphate, and optimum activity was observed with 10 µM pyridoxal phosphate.

3) Histidine Transport and Effects of Inhibition of Transport. The rate of uptake of ^{3}H-histidine by purified mast cells was constant with time (uptake by 90 min, 15.8 pmol/10^6 cells) and was proportional to cell number. Uptake was not observed with cells kept on ice and was blocked by glutamine, a substrate of the recently described histidine/glutamine transport (N) system (Kilberg et al., J. Biol. Chem. 255: 40, 1980) but not by methylaminoisobutyric acid, which is not a substrate for this system. The rate of histidine uptake by peritoneal cells depleted of mast cells was less than 5% of that observed with mast cells.

Of the compounds tested for their effects on histidine transport and histamine synthesis, L-glutamine, L-tryptophan, 2-fluorohistidine, β-thienylalanine, and, in high concentrations, α-methylDOPA inhibited decarboxylation as well as uptake of L-histidine in a dose-dependent fashion. They did not, however, inhibit the histidine decarboxylase activity of cell extracts of the soluble gastric histidine decarboxylase preparation. The decarboxylase inhibitors, α-aminohistidine and Brocresine, inhibited decarboxylase activity without interfering with L-histidine uptake, whereas α-methylhistidine inhibited both uptake and histamine synthesis in intact cells and histidine decarboxylation in the cell and gastric extracts. Methylaminoisobutyric acid inhibited neither uptake nor decarboxylation of histidine.

4) Effects of Disruption of Histamine-Containing Cells from other Tissues. The loss of histamine synthetic activity upon cell disruption was observed with mixed cell suspensions from peritoneal and pleural cavity as well as with the purified mast cells from rat and dog tissue. The loss of activity with partially purified preparations of ECL cells from rat gastric mucosa was, however, much less than that observed in rat and dog mast cells.

Significance to Biomedical Research: The studies indicate that histidine is transported into mast cells by the recently characterized system N, which cotransports histidine and glutamine. By comparison with that in peritoneal cells, uptake of histidine into mast cells occurs at a particularly rapid rate. At least under the conditions of the in vitro experiments, blockade of histidine uptake may impair histamine synthesis in the intact mast cell. This may

provide an additional mode of regulating histamine synthesis, although this remains to be proven by experiments *in vivo*. As is evident in the present study, significant loss of enzyme activity occurs upon homogenization of tissue mast cells. Although we are uncertain as to the mechanism(s) responsible for loss of activity, the intact mast cell does have a high capacity for histamine synthesis, and measurement of enzyme activity in tissue extracts may not be a meaningful reflection of actual histidine decarboxylase activity *in vivo*.

Proposed Course: The time course of changes in histamine synthesis and histidine uptake following mast cell degranulation will be studied in short-term culture to see if histamine stores are replenished and how this process is affected by suicide inhibitors of histidine decarboxylase such as α-fluoromethylhistidine. The kinetic parameters of histidine uptake by mast cells will be compared with those of other mammalian cells to determine whether or not rapid histidine uptake is unique to mast cells.

Publications:
Almeida, A.P., Flye, W., Deveraux, D., Horakova, Z., and Beaven, M.A.: Distribution of histamine and hiistaminase (diamine oxidase) in blood of various species. Comp. Biochem. Physiol. 67C; 187-190, 1980.

Moss, J., Fahmy, N.R., Sunder, N., and Beaven, M.A.: Hormonal and hemodynamic profile of an anaphylactic reaction in man. Circ. Res. 63: 210-213, 1981.

Beaven, M.A.: Editorial: Anaphylactoid reactions to anesthetic drugs. Anesthesiology, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00621-06 CM

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Reversible Inhibition of Cell 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:	Barbara M. Bayer	Staff Fellow	CM NHLBI
OTHER:	Michael A. Beaven	Head, Section on Cellular Pharmacology	CM NHLBI

COOPERATING UNITS (if any)

None

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Cellular Metabolism

SECTION
Cellular Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Marked increases in the rate of uptake of substrates (methylaminoisobutyric acid, MeAIB, and aminoisobutyric acid, AIB) of the Na^+ energy-dependent System A occurred during exponential growth in several cell culture lines and in thymus lymphocytes after stimulation with mitogenic agents or amino acid deprivation. Irrespective of the cell type or mechanism of stimulation, indomethacin and other anti-inflammatory drugs suppressed this increase without altering the affinity of the uptake system for the MeAIB or Na^+ ions. This inhibition was observed in thymus lymphocytes removed from rats given pharmacological doses of indomethacin. The early part of the cell cycle appears to be uniquely sensitive to the action of anti-inflammatory drugs in that, once DNA synthesis has commenced in cell cultures, indomethacin no longer suppresses mitogenesis. The data suggest that the Na^+-dependent components of MeAIB transport in both the thymus lymphocytes and cell culture lines share common features and interact with nonsteroidal anti-inflammatory drugs in a similar manner.

PHS-6040
(Rev. 2-81)

Project Description

Objectives: We have shown that nonsteroidal anti-inflammatory drugs inhibit, in a noncytotoxic manner, the growth of transplantable tumors in mice as well as rat hepatoma (HTC) and human diploid fibroblast cells in culture. The cells accumulate in the G_1 phase of the cell cycle and can be held in this state for prolonged periods (4-9 days) without loss of viability. In general, the ability of these drugs to suppress culture growth parallels their potency as anti-inflammatory agents, but not their ability to inhibit prostaglandin synthesis. After removal of the drug, all of the cells (> 98%) resume growth in synchrony through at least one cell cycle (see last year's and previous reports Z01 HL 00621-05 CM).

Last year, we reported that specific changes in amino acid transport accompany these events in HTC cell cultures. The transport of MeAIB and AIB through System A was inhibited by indomethacin and other nonsteroidal anti-inflammatory drugs, whereas the transport of leucine and BCH by System L was slightly stimulated. The effect on System L was immediate and that on System A was time- and dose-dependent. Other transport systems (nucleotides and deoxyglucose) were not inhibited by the anti-inflammatory drugs (Bayer et al., 1980).

The present studies were undertaken to see if indomethacin and other anti-inflammatory drugs have similar inhibitory effects on the Na^+-dependent component of MeAIB transport in both transformed and nontransformed cell culture lines and freshly isolated cells (rat thymocytes) and if such effects are apparent after pharmacological doses of indomethacin. Classical kinetic approaches were used to analyze the effects of anti-inflammatory drugs on the various components of amino acid transport.

Methods Employed: Procedures for cell cultures, the preparation of thymus lymphocytes, and measurement of amino acid uptake were described in detail in last year's report (Z01 HL 00621-05 CM) and in the publications listed below. The following describe differences in procedures from those of last year.

1) Preparation of cells. HTC and human fibroblasts (Kinser) cells were obtained from Dr. Vincent Manganiello (this laboratory) and BRL-T rat hepatocytes (cloned from 5-week-old Buffalo rats) from Dr. George J. Todaro (NCI). Rat thymus lymphocytes were separated by filtration through fine mesh nylon (Nitex, 40 μM mesh) and were suspended to give a final concentration of 0.2-0.3 x 10^8 cells/ml, unless indicated otherwise. Cell viability was assessed by fluorescence microscopy after staining with ethidium bromide and fluorescein diacetate.

2) Amino acid transport in thymic lymphocytes. Thymocyte suspension (60 μl) was added to an Eppendorf polypropylene microfuge tube (1.5 ml) and equilibrated at 37°C for 10 min prior to addition of 60 μl of Dulbecco's phosphate-buffered medium containing ^{14}C-labeled MeAIB (100 nCi, 10 μM) and unlabeled MeAIB where indicated. At the end of the incubation, the cells were separated by centrifugation through fetal calf serum in a Beckman microfuge. The supernatant was aspirated, and the conical end with the cell pellet was cut and assayed for radioactivity. All determinations were carried out in quadruplicate.

In experiments where cells were incubated in a different medium or exposed to indomethacin or concanavalin A before measurement of amino acid uptake, the cells were separated by centrifugation at 1000 x g for 1 min, washed, and were then resuspended in HEPES buffered Hanks' salt solution with albumin. Similar procedures were used when transferring cells to a Na^+-free medium. The results were corrected for nonspecific amino acid uptake (< 30 dpm/10^6 cells), which was determined by keeping cell suspensions on ice and using chilled reagents.

3) Analysis of data. In both cultured cells and thymus lymphocytes, the uptake of MeAIB best fitted two Michaelis-Menten components and computations were based on this assumption. Accordingly, data were analyzed by least square fit of the theoretical equations for the sum of two parabolas when V was plotted against S or one parabola when V was plotted against V/S according to the Woolf-Augustinson and Hofstee (Bayer et al., 1980).

4) Definition of amino acid transport systems. These systems have been defined by Christensen and co-workers in several mammalian cells using nonmetabolizable neutral amino acids, e.g., α-aminoisobutyric acid (AIB), α-methylaminoisobutyric acid (MeAIB), and aminobicycloheptane-2-carboxylic acid (BCH). The A system, which is Na^+- and energy-dependent, has an affinity for AIB and MeAIB and most neutral amino acids; a Na^+-dependent ASC system, which has an affinity for nonmethylated amino acids (it does not take up MeAIB); and an L system, which is not Na^+- or energy-dependent, has a high affinity aspartate-preferring systems have also been described for the transport of basic and acidic amino acids. Recently, another Na^+-dependent transport system (the N system) has also been shown to exist in mammalian cells possessing a high degree of selectivity for glutamine, histidine, and asparginine.

Major Findings: Cytostatic activity of indomethacin and structurally closely related compounds in cell cultures. In BRL-T rat hepatoma cultures, in the presence of < 3% serum, indomethacin was cytostatic at levels near those observed in plasma after pharmacological doses of drug (ED_{50}, 50 μm). In HTC cultures, the cytostatic concentration of indomethacin was generally higher than levels observed therapeutically, although a major proportion (80%) of the drug was bound to serum in the culture medium. Compounds resembling structural fragments of indomethacin, i.e., 5-methoxy-methylindoleacetic acid, chlorobenzoic acid, or indoleacetic acid, were not cytostatic, nor was inhibition observed with the anti-inflammatory steroid drug, dexamethasone.

Effect of indomethacin on amino acid transport in 3 cell culture lines. During exponential growth, a low and a high affinity component of the MeAIB transport was observed in all cell lines tested. The high affinity component was also present in confluent cultures of rat hepatoma (HTC) cells, whereas in nontransformed human fibroblasts the high affinity component largely disappeared when the cultures reached confluency. Only the high affinity component was Na^+-dependent. The Na^+-independent component of a much lower affinity accounted for only 8-10% of the total transport at low concentrations of MeAIB (0.01 mM).

In all cells, 0.4 mM indomethacin selectively inhibited the high affinity component producing a decrease in V_{max} with no change in the affinity (K_m) for the amino acid. The apparent K_m of the high affinity component for Na^+ (∿ 45 mM) was also unaltered in the presence of indomethacin.

The effects of indomethacin were reversible. In both fibroblasts and HTC cells, a return of the high affinity component was observed as early as 1 hr after removal of drug and its magnitude was equal to that of controls (no drug added) within 6 hr. These changes were attributable to increases in V_{max} and not K_m.

Exposure to indomethacin for 4 hr did not inhibit the recently characterized Na^+-dependent glutamine uptake system (N) in human fibroblast cultures.

Uptake of MeAIB by rat thymic lymphocytes: Effects of anti-inflammatory drugs, concanavalin A, or amino acids. The rate of MeAIB uptake was proportional to cell concentrations up to 2.4 x 10^6 cells/120 μl and was constant up to 20 min. There was little uptake when cells were kept at 4^oC. Variations in the rate of uptake (V) with different MeAIB concentrations (S) in the absence and presence of Na^+ indicated that a Na^+-dependent component with a high affinity for MeAIB (K_m 0.2 mM) and a Na^+-independent one with a low affinity for MeAIB (K_m ∿ 15 mM) were present.

When thymus lymphocytes were incubated in media supplemented with essential amino acids, there was little change in the rate of MeAIB uptake with time nor was uptake much affected by indomethacin except at later times (after 3.5 hr) when it declined. During incubation for 5 hr without amino acids, the rate of MeAIB uptake increased 300% and this was prevented by indomethacin. The major change in the absence of amino acid was an increase in V_{max} of the high affinity Na^+-dependent component with no change in K_m. The high affinity component was much less prominent in both the indomethacin-treated and nonincubated lymphocyte suspensions and the values for the kinetic parameters were similar. Phenybutazone, meclofenamate, and acetylsalicylic acid, like indomethacin, prevented the inductio of MeAIB uptake in the absence of amino acids.

Stimulation of MeAIB uptake concanavalin A in the presence of amino acids was also prevented by indomethacin. The increase in MeAIB uptake was of gradual onset and was usually apparent 60 min after the addition of concanavalin A. By 2 hr, uptake was consistently increased (157 ± 40%, mean ± S.E., n = 7), but when 0.4 mM indomethacin was present the increase was only 27 ± 17%.

Significance to Biomedical Research: Although many therapeutic effects of the nonsteroidal anti-inflammatory drugs have been ascribed to the inhibition of prostaglandin synthesis, and this action has been given specific emphasis in recent years, other actions may be equally decisive in the immunosuppressive activity of these drugs.

Our work shows that the nonsteroidal anti-inflammatory drugs inhibit both the growth of cultured cells and lymphocyte transformation by interfering with mechanisms essential for expression of the A transport system early in the cell cycle. These effects may be of therapeutic significance and have potential use in studies of the cell cycle.

Proposed Course: Project will be terminated upon departure of principal investigator and will probably be pursued by this investigator at an outside institution.

Publications: Bayer, B. M., Lo, T. N., and Beaven, M. A.: Anti-inflammatory drugs alter amino acid transport in HTC cells. J. Biol. Chem. 255: 8784-8790, 1980.

Beaven, M. A. and Bayer, B. M.: Factors influencing the uptake and disposition of indomethacin-^{14}C in cell cultures. Biochem. Pharmacol. 29: 2055-2061, 1980.

Almeida, A. P., Bayer, B. M., Horakova, Z. and Beaven, M. A.: Influence of indomethacin and other anti-inflammatory drugs on mobilization and production of neutrophils: Studies with carrageenan-induced inflammation in rats. J. Pharmacol. Exp. Ther. 214: 74-79, 1980.

Bayer, B. M. and Beaven, M. A.: Cytostatic activity of pharmacological concentrations of indomethacin in cell cultures and inactivity of closely related compounds, Biochem. Pharmacol. 30: 807-809, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00622-04 CM

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Regulation of Cyclic Nucleotide Metabolism

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

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	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI
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TOTAL MANYEARS: 2.7	PROFESSIONAL: 1.2	OTHER: 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)

1) Choleragen activates adenylate cyclase by ADP-ribosylating an arginine-like residue in the regulatory component of the cyclase. The toxin must be reduced by thiol to be active; its activity was enhanced by thiol:protein disulfide oxidoreductase. 2) GTP enhanced the activation of adenylate cyclase by choleragen by increasing ADP-ribosylation of the toxin substrate, enhancing stability of the ADP-ribosylated enzyme, and facilitating expression of the activated enzyme in the assay. 3) NAD:arginine ADP-ribosyltransferases were identified in human erythrocytes and rat liver and exhibited characteristics similar to the transferase from avian erythrocytes. The activity of the erythrocyte ADP-ribosyltransferases was enhanced > 10-fold by micromolar histones. At these concentrations, histones activated the enzyme without serving as ADP-ribose acceptors. Certain inorganic salts, with chaotropic salts being the most active, increased ADP-ribosyltransferase activity to the same extent as that observed with histones. Activation by salt resulted in the conversion of inactive oligomeric forms of the transferase to active protomeric forms. The activated transferase in the presence of salt no longer responded to histones.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: To study the regulation of cyclic nucleotide metabolism. The adenylate cyclase system is responsible for the synthesis of cyclic AMP; it has been shown to be composed of multiple components. Studies on the activation of adenylate cyclase by choleragen (cholera toxin) defined the G/F subunit of the cyclase system and the importance of G/F factor and guanine nucleotides in cyclase action. Studies on analogies to the choleragen-catalyzed reaction in animal cells revealed that erythrocytes possess an ADP-ribosyltransferase with catalytic properties similar to the toxin. Our investigations on this enzyme defined its regulation by histone and its activation by chaotropic salts.

Methods Employed: Assays. ADP-ribosyltransferase and NAD glycohydrolase activities were determined by our published methods. ADP-ribosylhistone or ADP-ribosyl protein formation was determined in assays containing [adenine-U-^{14}C]NAD by our published method except that 20% trichloroacetic acid was used.

The turkey erythrocyte transferase, purified by our published procedure, showed one major band on sodium dodecyl sulfate-polyacrylamide gels. The human erythrocyte ADP-ribosyltransferase was purified through the carboxymethyl cellulose chromatography step.

Major Findings: 1) Activation of Choleragen by Thiol:Protein Disulfide Oxidoreductase. Choleragen activates adenylate cyclase by catalyzing the NAD-dependent ADP-ribosylation of a regulatory protein of the cyclase system. The ability of the toxin to activate adenylate cyclase appears to be a specific property of the A subunit of the toxin. The A subunit, however, which consists of two polypeptides, A_1 and A_2, linked through a single disulfide bond, appears to be relatively inactive in the ADP-ribosylation assay; rather, the catalytically active A_1 peptide must be released by cleavage of the disulfide bond linking it to A_2. *In vivo*, this event is believed to occur following binding of the complex to the cell surface. It is not clear whether reductase of the A_1-A_2 disulfide linkage is enzymatic or nonenzymatic. Jack Dixon and co-workers have reported the isolation of an enzyme that catalyzes disulfide exchange. Since this protein is found in the plasma membrane, it would appear to be a prime candidate to catalyze the activation of choleragen.

The ability of a purified thiol:protein disulfide oxidoreductase from bovine liver to activate choleragen in the presence of thiol was examined; the activity of choleragen was determined by monitoring its ability to catalyze the ADP-ribosyaltion of arginine; in the absence of cellular components, arginine and other guanidino compounds serve as ADP-ribose acceptors.

The activities of choleragen and its A protomer in the presence of limiting concentrations of thiol were increased > 20-fold by thiol:protein disulfide oxidoreductase. Oxidoreductase decreased the concentration of thiol necessary for an equivalent activation of the toxin and, in the presence of limiting concentrations of glutathione or dithiothreitol, increased the rate of activation of choleragen and its A protomer. The ability of oxidoreductase preparations to activate choleragen cochromatographed with oxidoreductase protein on gel permeation columns and was proportional to the concentrations of oxidoreductase in the assay.

The data are consistent with the hypothesis that the thiol:protein disulfide oxidoreductase could play a role in the reduction of choleragen and release of the catalytically active A_1 peptide. A lag has been observed in activation of cyclase by toxin in all systems that have been examined. This lag has, in part, been attributed to the time necessary for internalization of the toxin and for the A_1 peptide to locate the adenylate cyclase component which serves as the ADP-ribose acceptor. Although there is no evidence that reduction of the disulfide bond linking A_1 and A_2 is rate-limiting, clearly this possibility exists. The lag thus depends in part on the ability of the oxidoreductase to release the A_1 peptide.

2) Effects of Nucleoside Triphosphates on Choleragen-Activated Brain Adenylate Cyclase. To investigate the effects of nucleoside triphosphates on the activation of adenylate cyclase by choleragen and on the stability and catalytic function of the choleragen-activated enzyme, we treated samples of particulate preparation from bovine brain successively in three separate incubations with extensive washing between each step. In incubation I, choleragen and NAD were present to activate the adenylate cyclase. During incubation I, the toxin catalyzed the NAD-dependent ADP-ribosylation of a 42,000 dalton membrane protein believed to bind GTP. In incubation II, stability of the adenylate cyclase to thermal denaturation was examined. Finally, adenylate cyclase activity was assayed with ATP or adenylyl imidodiphosphate [App(NH)p] as the substrate. Even when assays contained an optimal concentration of GTP, nucleoside triphosphate (plus a regenerating system) was required in incubation I for maximal choleragen activation; in order of effectiveness, GTP > ITP >> ATP $\geq$ CTP = UTP. During incubation II (at 30°C), activity of the choleragen-treated fractions was essentially completely stable when 100 μM GTP (plus a regenerating system) was present. ITP and ATP were less effective. Activation produced by guanylyl imidodiphosphate was more stable than that resulting from choleragen, GTP, and NAD. After activation of membranes with choleragen, NAD, and GTP, nucleoside triphosphate plus a regenerating system was necessary to express maximal activity. In order of effectiveness, GTP > ITP >> ATP $\geq$ CTP = UTP. It appears that GTP, which was effective in micromolar concentrations, plays an important role not only in the activation of adenylate cyclase by choleragen but also in the stabilization and expression of the catalytic function of the activated enzyme.

3) Histone-Dependent and Histone-Independent Forms of an NAD:Arginine ADP-Ribosyltransferase from Human and Turkey Erythrocytes. The ADP-ribosyltransferase from turkey erythrocytes appeared to exist both in high activity, histone-independent and low activity, histone-dependent forms. The histone-independent state occurred in the presence of salt, the histone-dependent state in its absence. At low salt concentrations, the activity of the transferase with agmatine as acceptor was < 10% that observed in the presence of 200 mM NaCl. In the absence of salt, ADP-ribosylation of agmatine was stimulated > 10-fold by < 1 μM histones and activity approached that observed with high salt. Maximal effects of histones were observed at 10 μg-20 μg/ml. At 10 μg/ml, ADP-ribosylation of agmatine was stimulated by histone and histone fractions to a significantly greater extent than it was by other purified proteins. In the presence of agmatine, stimulation of [carbonyl-^{14}C]nicotinamide release from [carbonyl-^{14}C]NAD by histone was coupled to [adenine-U-^{14}C]ADP-ribosylagmatine formation with [adenine-U-^{14}C]NAD as substrates. Stimulation

of ADP-ribose-agmatine synthesis by histone was observed at histone concentrations which were much lower than those necessary for the histone to serve as an effective substrate for the transferase. Thus, it appears that histones act as allosteric activators of the enzyme from turkey erythrocytes. Histone also activated the highly purified ADP-ribosyltransferase from human erythrocytes. Enzyme activity was increased in the presence of salt and was then relatively independent of histones. DNA was not required to observe the stimulation of ADP-ribosylation by histone; incubation of the transferase and histone with DNase did not significantly decrease enzymatic activity. Additional DNA in the assay decreased the effect of histone. The erythrocyte ADP-ribosyltransferase from diverse species thus appears to exist in two forms, one which is dependent on histones for activity and one which in the presence of salt has high intrinsic activity and is independent of histone. The fact that the active form(s) of the transferase generated in the presence of salt or histone has similar catalytic activity suggests that these forms of transferase may be identical. It would appear that the enzymatic activity of transferase from different species may be controlled by histones.

4) Activation of an NAD:Arginine ADP-Ribosyltransferase from Turkey Erythrocytes by Chaotropic Salts. The NAD:arginine ADP-ribosyltransferase isolated from turkey erythrocytes was enhanced by several inorganic salts. Chaotropic salts were most effective with $SCN^- > Br^- > Cl^- > F^- > PO_4$; the activity of the salt corresponded to its position in the Hofmeister series for the salting out of englobulins. The nature of the monovalent cation (Na, K, or Li) had little influence on the effectiveness of a given anion. Activation did not result from stabilization alone, since under the assay conditions at 30°C the reaction rate was linear both in the absence and presence of NaCl. In the absence of NaCl, double reciprocal plots gave limiting slopes corresponding to apparent Michaelis constants of 3.8 and 50 mM, respectively, for arginine methyl ester. The standard V versus substrate plot was consistent with the presence of two enzyme species. In the presence of NaCl, the double reciprocal plot was linear extrapolating to give an apparent K_m of 1.3 mM . Other guanidino derivatives also stimulated [carbonyl-^{14}C]nicotinamide release; classical Michaelis-Menten kinetics were observed in the presence of NaCl. Substrates possessing positively charged groups near the guanidino moiety showed significantly more activity: arginine methyl ester > agmatine $\geq$ arginine > guanidinopropionate $\geq$ guanidine; creatine was inactive. In the presence of NaCl, the K_m for [carbonyl-^{14}C]NAD was reduced slightly. With ovalbumin as the ADP-ribose acceptor, low concentrations of NaCl increased the release of [carbonyl-^{14}C]nicotinamide from [carbonyl-^{14}C]NAD and the incorporation of [adenine-U-^{14}C]ADP-ribose from [adenine-U-^{14}C]NAD into protein; at higher concentrations of NaCl, [carbonyl-^{14}C]nicotinamide release but not [adenine-U-^{14}C]ADP-ribose incorporation was increased. In contrast, with agmatine as ADP-ribose acceptor, NaCl increased ADP-ribosylation in parallel with [carbonyl-^{14}C]nicotinamide release. In the absence or presence of 200 mM NaCl, the ratio of [adenine-U-^{14}C]ATP-ribose coupled to agmatine to [carbonyl-^{14}C]nicotinamide released using [adenine-U-^{14}C]NAD and [carbonyl-^{14}C]NAD, respectively, as substrates was $\sim$ 0.90.

In the presence of NaCl and propylene glycol, a large fraction of the ADP-ribosyltransferase exhibited a sedimentation coefficient and a K_{av} on Sephadex G-200 similar to those of chymotrypsinogen (MW $\sim$ 25,000). As noted previously, the transferase shows a molecular weight of 28,300 by sodium

dodecyl sulfate-polyacrylamide gel electrophoresis. In the absence of NaCl, sedimentation of the transferase was consistent with the presence of multiple associated species and was eluted in the void volume of a Sephadex G-20 column. Assays run under conditions identical to those used to examine the sedimentation and gel permeation properties of the transferase showed that NaCl increased the activity of the transferase > 10-fold. These data are consistent with the highly purified erythrocyte transferase existing as relatively inactive, high molecular weight oligomers which are converted by chaotropic salts to high activity protomeric species.

5) Identification of NAD:Arginine ADP-Ribosyltransferases in Human Erythrocytes and Rat Liver. An NAD:arginine ADP-ribosyltransferase was previously identified as turkey erythrocytes. Using a purification procedure similar to the one used for isolation of the turkey erythrocyte enzyme, similar transferases were identified in 100,000 x *g* supernatant fractions from human erythrocytes and rat liver.

Significance to Biomedical Research: The pulmonary and cardiovascular systems are affected under physiological and pathological conditions by extracellular agents such as hormones and toxins. The lung, in particular, is exposed through the tracheobronchial tree to a variety of bacterial and toxic agents. To be effective, some of these agents must interact with a cellular receptor, and a substantial number of these agents exert their effects by altering the steady-state levels and localizations of cyclic nucleotides within the cell. A number of pharmacological agents have been in use which override aberrant physiological control to the benefit of the patient; these agents have been designed to interact with specific cellular receptors. By using cultured cells, model systems and purified preparations, it may be possible to simplify and define the factors critical to cyclic nucleotide metabolism. These models can then be used to understand the controls which operate in the more complex pulmonary and cardiovascular system.

Proposed Course: 1) To define the mechanism of activation of adenylate cyclase by hormones and toxin. Studies on choleragen action have enabled investigators to define the role of ADP-ribosylation in the activation of adenylate cyclase; choleragen-catalyzed ADP-ribosylation has enabled investigators to label the G/F component of the cyclase system. Choleragen will continue to be used as probe to define the G/F subunit and its interaction with guanine nucleotide and hormone receptors. 2) To define the mechanisms for regulation of ADP-ribosylation by endogenous ADP-ribosyltransferases. Prior studies in this laboratory have demonstrated an ADP-ribosylation reaction in animal cells that mimics the reaction catalyzed by toxin. In contrast to toxin, however, this enzyme is stimulated by histones. The role of histones in the regulation of ADP-ribosylation will be defined.

Publications: Moss, J., Stanley, S.J., and Watkins, P.A.: Isolation and properties of an NAD- and guanidine-dependent ADP-ribosyltransferase from turkey erythrocytes. J. Biol. Chem. 255: 5838-5840, 1980.

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Moss, J. and Stanley, S.J.: Histone-dependent and histone-independent forms of an ADP-ribosyltransferase from human and turkey erythrocytes. Proc. Natl. Acad. Sci. U.S.A., in press.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00625-03 CM

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
ADP-Ribosyltransferases: Characterization of their Substrates and Factors that Control their Activity

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

PI:	Paul A. Watkins	Research Associate	CM	NHLBI
	Joel Moss	Head, Section on Molecular Mechanisms	CM	NHLBI
	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Cellular Metabolism

SECTION
Metabolic Regulation

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Major labeled peptides of <u>M_r = 42,000 and 47,000</u> were observed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis of <u>human skin fibroblast</u> membranes incubated with <u>choleragen</u> and <u>^{32}P-NAD</u>. Prior incubation of intact fibroblasts with choleragen blocked specifically the subsequent <u>^{32}P-ADP-ribosylation</u> of these two proteins in cell particulate fractions. The effect of choleragen was dependent on time, temperature, and toxin concentration. Neither the choleragen A subunit nor the B subunit nor the A_1 peptide could replace the holotoxin in cell incubations. Inhibition of subsequent labeling by prior exposure of cells to choleragen was correlated with increased <u>cellular cAMP</u>. Incubation of fibroblasts with prostaglandin E_1 and isoproterenol, which activate adenylate cyclase by different mechanisms, did not block subsequent labeling with choleragen and ^{32}P-NAD. The results suggest that proteins of M_r = 42,000 and 47,000 may be <u>in vivo</u> substrates for choleragen in human fibroblasts.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: Choleragen (cholera enterotoxin) catalyzes the NAD-dependent ADP-ribosylation of numerous proteins in broken cells. In intact cells, choleragen activates adenylate cyclase, presumably via ADP-ribosylation of the guanine nucleotide-binding regulatory component (G/F) of the cyclase. The purpose of this study was to identify those proteins in intact human fibroblasts which serve as substrates for the toxin and which may, therefore, be G/F or a subunit(s) of G/F. Cellular cAMP levels were measured to correlate ADP-ribosylation with cyclase activation.

Methods Employed: Particulate fractions were prepared from cultured human skin fibroblasts previously incubated with or without choleragen and incubated at 30°C with [^{32}P]NAD and fresh choleragen. Trichloroacetic acid-precipitated proteins were subjected to SDS-polyacrylamide gel electrophoresis and radioautography. Cellular cAMP was determined by radioimmunoassay.

Major Findings: When a particulate fraction of human skin fibroblasts was incubated with activated choleragen and ^{32}P-NAD, major labeled proteins of 42,000 and 47,000 daltons were observed on SDS-polyacrylamide gels. GTP enhanced labeling of these peptides. Prior incubation of cells with choleragen for 3 h prevented the subsequent labeling of both 42,000 and 47,000 dalton peptides. It seems most probable that labeling of the 42,000 and 47,000 dalton peptides was reduced or abolished because they had already been ADP-ribosylated during exposure of the intact cells to choleragen, i.e., that these peptides represent substrates for the choleragen-catalyzed reaction in intact fibroblasts.

Decreased labeling of both 42,000 and 47,000 dalton proteins was seen in particulate fractions from cells incubated with choleragen for as little as 20 min. After 60 min of choleragen treatment of cells, subsequent labeling of the 42,000 dalton species was completely prevented. In addition to the 42,000 and 47,000 proteins, a labeled peptide of intermediate M_r was frequently observed; labeling of this protein, which was unaffected by prior incubation of cells with choleragen, was seen best when labeling of the 42,000 and 47,000 dalton protein was prevented. Corresponding to the decreased labeling of the 42,000 and 47,000 dalton proteins was a rise in the cellular cAMP content during incubation of cells with choleragen.

Labeling of the two peptides in particulate fractions was inversely related to the choleragen concentration to which cells were exposed. When cells were incubated for 1 h with increasing amounts of choleragen, decreasing incorporation of ^{32}P-NAD into particulate proteins was observed, as well as increasing cellular cAMP levels. Labeling of particulate proteins was also dependent on the choleragen concentration during incubation with ^{32}P-NAD. Incubation of labeled particulate proteins with snake venom phosphodiesterase released radioactivity, all of which migrated with 5'-AMP on thin-layer chromatography, indicating that the labeled peptides were ADP-ribosylated rather than poly(ADP-ribosylated).

Incubation of fibroblasts with choleragen at 4°C (rather than 37°C) did not prevent the subsequent labeling of particulate proteins and did not increase

the cellular cAMP content of these cells. The addition of choleragen antitoxin to cell incubations also prevented the effects of choleragen on cAMP and on subsequent labeling of particualte peptides. No change in the effect of choleragen or in the mobilities of the labeled peptides was observed when all procedures were carried out in the presence of 50 μM phenylmethyl sulfonyl fluoride.

The effect of choleragen on ADP-ribosylation of fibroblasts was not reproduced by either the A or B subunits of cholera toxin. Similarly, incubation of cells with the A_1 peptide of cholera toxin did not prevent subsequent labeling of the 42,000 and 47,000 dalton proteins, even though the activated A subunit catalyzed the ^{32}P-ADP-ribosylation of these two proteins in membrane preparations. Although none of the choleragen subunits prevented subsequent labeling of particulate proteins, slight elevations in cellular cAMP levels were observed. Incubation of cells with prostaglandin E_1 or isoproterenol, which increase intracellular cAMP in these fibroblasts by mechanisms different from that of choleragen, did not prevent the labeling of particulate proteins with choleragen and ^{32}P-NAD.

Significance to Biomedical Research: Many metabolic processes in mammalian tissues, such as heart, lung, adipose, etc., are regulated by a hormone-dependent adenylate cyclase system. Currently, mammalian cyclases are thought to consist of three components--hormone receptor (R), catalytic subunit (C), and guanyl nucleotide-binding regulatory subunit (G/F). Certain activators of adenylate cyclase, e.g., fluoride, guanylylimidodiphosphate, and choleragen, interact directly with G/F, which subsequently causes increased catalytic activity of C. Hormones and pharmacological agents such as epinephrine and isoproterenol first bind to R; R must interact with G/F for activation of C to occur. Therefore, detailed knowledge of the G/F protein is essential for an understanding of regulation of adenylate cyclase. Choleragen catalyzes the ADP-ribosylation of G/F (or subunits of G/F), as well as other proteins in vitro. By using the methods employed in this study, it was possible to identify proteins which are the likely in vivo choleragen substrates. Once identified, these proteins can be ^{32}P-ADP-ribosylated and monitored isotopically.

Proposed Course: To study the effect of cell growth conditions, cell age, hormones, and pharmacological agents on the level of G/F in fibroblasts.

Publications: Watkins, P.A., Moss, J., and Vaughan, M.: ADP ribosylation of membrane proteins from human fibroblasts: Effect of prior exposure of cells to choleragen. J. Biol. Chem. 256: 4895-4899, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00627-03 CM

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Interaction of GTP-Binding Proteins and Catalytic Component of Adenylate Cyclase

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

PI:	Alan J. Bitonti	Staff Fellow	CM	NHLBI
	Bruce I. Terman	Staff Fellow	CM	NHLBI
	Joel Moss	Head, Section on Molecular Mechanisms	CM	NHLBI
	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI

COOPERATING UNITS (if any)

None

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TOTAL MANYEARS: 1.9	PROFESSIONAL: 1.9	OTHER: 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 interactions of the catalytic (C) and GTP-binding (G/F) proteins of adenylate cyclase are important in understanding the mechanisms by which hormones, cholera toxin and other agents regulate adenylate cyclase. We are attempting to isolate and characterize C and G/F from various animal tissues. A variant strain of S49 mouse lymphoma cells which lack the GTP-binding protein allows us to assess the progress of purification of G/F. We have also used the S49 cells to identify factors which can be used to stabilize C.

Separation of C and G/F from bovine brain has been achieved using a new zwitterionic detergent CHAPS. The method for preparation of C and G/F is an improvement over existing procedures.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: Hormone-sensitive adenylate cyclase consists of at least three protein components: a hormone receptor, the catalytic unit (C), and a guanyl nucleotide-binding protein (G/F). As a prerequisite to understanding the interactions between these proteins, we are attempting to purify C and G/F from various animal tissues and to reassemble the proteins in artificial lipid membranes.

Methods Employed: Human erythrocyte membranes are prepared by hemolysis and extensive washing in hypotonic buffers at neutral pH. The purified membranes are solubilized with 10 mM CHAPS followed by centrifugation at 100,000 x g for 60 min. G/F is determined by reconstitution with variant S49 lymphoma cell (AC^-) membranes which are genetically deficient in G/F (Ross et al., J. Biol. Chem. 252: 5761, 1977). The lymphoma cell membranes have no demonstrable adenylate cyclase activity in the standard assay unless combined with a detergent extract of cell membranes that contain G/F. After solubilization of AC^- cell membranes with 13 mM CHAPS, the physical properties of C were investigated using gel filtration on Sepharose 6B and sucrose density gradient centrifugation (5-20% linear sucrose gradients). The stability of C from AC^- cell membranes was studied by incubation of C at 30°C in the presence or absence of potential stabilizing guanine and adenine nucleotides.

C and G/F were solubilized from bovine brain cortex with 13 mM CHAPS, 12 mM sodium deoxycholate, 15 mM sodium cholate, or detergent plus 0.5 M ammonium sulfate, and adenylate cyclase activity was determined in each extract. The responses of the various extracts to effects of phospholipids, Gpp(NH)p, and calmodulin were also compared. CHAPS-solubilized bovine brain adenylate cyclase was chromatographed on Ultrogel AcA34 to separate C and G/F.

Adenylate cyclse activity was assayed by the procedure of Salomon et al. (Anal. Biochem. 58: 541, 1974).

Major Findings: The catalytic unit of adenylate cyclase (C), in intact or CHAPS-solubilized AC^- membranes, is inactivated by incubation at 30°C and can be stabilized at 30°C by ATP or App(NH)p in the absence of divalent cations. As AC^- membranes lack the GTP-binding protein (G/F), the stabilizing effect is presumed to be directly on C. Gpp(NH)p, ADP-β-S, and AMP-S were not effective in stabilizing C.

The hydrodynamic properties of C in 13 mM CHAPS were very similar to those reported for C solubilized with other detergents. A Stokes radius of 7.4 nm was obtained when AC^- membrane extracts were chromatographed on Sepharose 6B in the presence of 13 mM CHAPS. The sedimentation coefficient ($S_{20,w}$) for C obtained with sucrose density gradient centrifugation was 8.8.

CHAPS was found to be a better detergent than either sodium cholate, sodium deoxycholate, or one of these detergents plus 0.5 M ammonium sulfate for solubilization of bovine brain adenylate cyclase. Solubilization with CHAPS resulted in a significantly higher specific activity of adenylate cyclase, and since no $(NH_4)_2SO_4$ was present the enzyme activity could be more accurately determined without dilution. C and G/F were completely separated by gel filtration of

CHAPS-solubilized bovine brain membranes. This method permits the simple and rapid preparation of separate subunits (C and G/F) of adenylate cyclase for further studies.

Significance to Biomedical Research: Many hormones, drugs, and other agents exert their effects on cells by modifying cyclase activity, but the molecular mechanisms by which this important enzyme is regulated remain to be fully elucidated.

Proposed Course: To further define the molecular interactions between the GTP-binding protein (G/F) and the catalytic unit (C) of adenylate cyclase.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 HL 00630-02 CM

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Metabolism of Lipids in Human Fibroblasts and Muscle Cells Grown in Culture

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

PI:	Joel Avigan	Research Chemist	CM	NHLBI

COOPERATING UNITS (if any)

Medical Neurology Branch, NINCDS

LAB/BRANCH
Cellular Metabolism

SECTION
Metabolis Regulation

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.9	PROFESSIONAL: 0.6	OTHER: 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)

Carnitine-depleted cultures of human skin fibroblasts and of rat and human skeletal muscle cells were prepared. Their greatly depressed capacity to oxidize fatty acids was effectively restored on adding L-carnitine to the incubation medium. Carnitine concentration as low as 2.5 x 10^{-8} M significantly stimulated the above process. Addition of carnitine to the depleted fibroblasts reduced incorporation of palmitate into glycerides. Consequently, carnitine seemed to counteract cellular accumulation of lipids. Dexamethasone increased palmitate oxidation independently of the effect of carnitine. Fatty acid oxidation in cultured fibroblasts from two patients with muscle carnitine deficiency responded normally to exogenous carnitine and dexamethasone. The results demonstrate a rationale for treating these patients with carnitine and glucocorticoids.

PHS-6040
(Rev. 2-81)

Project No. Z01 HL 00630-02 CM

Project Description

Objectives and Background: This is a continuation of project No. Z01 HL 00630-01 CM, concerned with control of fatty acid metabolism and with the role of carnitine therein in cultured fibroblasts and muscle cells derived from human controls and patients with muscle carnitine deficiency as well as in cultured rat skeletal muscle and HTC cells. While results described in last year's report show rather modest effects of carnitine and only when added at relatively high concentration, our subsequent experiments were done with carnitine-depleted cells and demonstrated almost complete dependence of fatty acid metabolism on exogenous carnitine.

Methods Employed: Monolayers of cultured fibroblasts or skeletal muscle cells were established by conventional methods using carnitine-free growth media. In a typical experiment, cells were incubated, usually for 20 to 24 hr, with or without additions such as L-carnitine or dexamethasone, they were washed and incubated again for 2-3 hr with a radio-labeled substrate, usually [^{14}C]palmitate. Incubation was terminated by acidification and the radioactive CO_2 was collected on alkali-wetted paper in specially designed plate covers and assayed for radioactivity.

Major Findings: It was shown that biological materials used to grow and maintain the various cell cultures, e.g., fetal calf serum and chicken embryo, extract contain significant amounts of carnitine that is taken up by the cells. Very exhaustive dialysis effectively removed carnitine from these fluids which consequently produced carnitine-depleted cells. Such cells were almost totally incapable of metabolizing fatty acids but retained their metabolic capacity with respect to other substrates. They presented, therefore, a useful model for study of the role of lipid as an energy source in mammalian cells. The capacity to metabolize fatty acids (palmitate, laurate, or hexacosanoate) was increased by adding L-carnitine to medium. Carnitine concentrations as low as 2.5×10^{-8} increased the rate of oxidation of labeled palmitate by 35%, and 10^{-4} M carnitine stimulated it 6- to 10-fold in human fibroblasts. Similar results were obtained with the other cells studied. It was shown that, in the absence of carnitine, fibroblasts incorporated increased amounts of labeled palmitate into di- and triglycerides. This observation may explain the lipid accumulation in muscles of patients with carnitine deficiency. Glucocorticoids have been successfully used in treatment of some of these patients. In the present study, dexamethasone increased palmitate oxidation in fibroblasts and muscle cells, either in the absence or in the presence of carnitine. Palmitate oxidation in cultured fibroblasts from two patients with muscle carnitine deficiency responded to exogenous carnitine and to dexamethasone to a degree similar to that seen in normal fibroblasts.

Significance to Biomedical Research: Disorders of fatty acid metabolism are associated with various metabolic diseases. Furthermore, the process of lipolysis followed by metabolism of released fatty acids is necessary for disposal of lipids and for supply of energy to the organism. Carnitine is an obligatory cofactor in mitochondrial metabolism of fatty acids. The *in vitro* model developed in the course of this work may facilitate gaining information on the role of fatty acid metabolism in mammalian cells and, in particular, on the effect of carnitine thereon.

Proposed Course of Study: It is being planned to study cells from additional patients with carnitine deficiency and other defects of fatty acid metabolism and to investigate possible abnormalities in the distribution of carnitine between such cells and medium containing serum from normals and patients.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00631-01 CM

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Cellular Distribution of Histamine and its Metabolizing Enzymes in Tissues: Studies with Microvascular Endothelial Cells

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

PI:	Audrey Robinson-White	Guest Worker	CM	NHLBI
OTHER:	Michael A. Beaven	Head, Section on Cellular Pharmacology	CM	NHLBI

COOPERATING UNITS (if any)

Dr. Robinson-White is a recipient of an NRSA postdoctoral fellowship.

LAB/BRANCH
Cellular Metabolism

SECTION
Cellular Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

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)

Purified microvascular endothelial cell preparations from guinea pig fat pad, heart, and brain were found to contain high histamine methyltransferase (HMT) activity. These cells appeared to account for most of the HMT activity in the whole fat pad, whereas in heart and brain the enzyme was not restricted to endothelial cells. The myocyte, for example, was an additional source of HMT activity in heart. HMT activity in the endothelial cells from fat pad and heart was the highest found so far in guinea pig tissues. In rat, a species in which diamine oxidase (DAO) is the major degradative enzyme for histamine, HMT activity was ~ 1% of that in the same preparations from guinea pig. DAO activity in the microvascular cell preparations from both species was 5 to 40 times than in the parent tissues. In addition to their ability to degrade serotonin and catecholamines, the microvascular endothelial cells may be an important site of inactivation of circulating histamine.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: This project reflects our past interest in the origin and distribution of histamine and its metabolizing enzymes, histidine decarboxylase (HbD), histamine methyltransferase (HMT) (EC 2.1.1.8), and diamine oxidase (DAO) (EC 1.2.6). Our phylogenetic studies last year (No. Z01 HL 00629-01 CM) showed that histamine first appeared in brain and gastric mucosa during vertebrate evolution. Our collaborative studies with investigators at Wadsworth V.A. Hospital, Los Angeles (see current report No. Z01 HL 00619-04), have shown that histamine and HdD activity are located in the gastric glands in mast cells in dog and man and in an APUD-type cell in rodents. The principal degrading enzyme, HMT, is located in the parietal cell. Other workers have reported recently that, in brain, histamine is located in a central core of neurones that pass through the primitive limbic system to forebrain structures as well as in mast cells along small blood vessels. In tissues other than brain and stomach, histamine appears only in terrestrial vertebrates, and most of this histamine is located in mast cells along small blood vessels and the mucosal linings of the respiratory and intestinal tract. This project was undertaken to identify the major sites of histamine degradation in the body, and we have focused our attention on the microvascular system.

Several lines of evidence suggest that the sites of histamine degradation are readily accessible to free histamine in the circulation. Upon intravenous injection, labeled histamine is rapidly inactivated (half-life in various species < 30 sec) and greater than 90% of the label can be recovered in tissues as metabolites within 2-5 min. Since the vascular endothelial cells in lung and other tissues are an important site of uptake and inactivation of serotonin and catecholamines, it seemed a reasonable possibility that these cells may contain histamine-inactivating enzymes as well. This report describes the preparations of purified microvessel and other cell preparations from different tissues of rat and guinea pig. Studies with these preparations show that HMT and, to a lesser extent, DAO activity is present in microvessel endothelial cells and accounts for a major portion of the histamine-degrading activity in some tissues.

Methods Employed: 1) Isolation of Fat Pad Microvessels. Epididymal fat pads from male rats and guinea pigs were dissected to remove large blood vessels (> 50 μM) and the tissue digested in medium supplemented with collagenase. The suspension was centrifuged, the supernatant fraction and the floating cake of fatty material were decanted, and the pelleted material was subjected to a series of washing and density gradient separation procedures as described by Robinson-White *et al*. (J. Pharmacol. Exp. Ther. 216: 125, 1981). The final preparation of microvessels was resuspended in 5 ml of medium.

2) Isolation of Myocardial Microvessels. Heart ventricular muscle (~ 1 g) was trimmed free of endocardium and epicardium. The tissue was minced in a McIlwain tissue chopper (1-mm cubes), suspended in Ca^{++}-free medium, and filtered through a 20-μm nylon gauze Nitex filter. The retained tissue was incubated with collagenase in a Ca^{++}-free medium, homogenized lightly in a Dounce glass homogenizer, and diluted with an equal volume of Ca^{++}-free medium. Microvascular cells were separated by the density gradient separation procedure described by Robinson-White (see ref. above).

3) Isolation of Cerebral Microvessels. These were prepared by a modification of the procedure of Williams et al. (J. Neurochem. 35: 374, 1980). Cortices were dissected from whole brain, trimmed free of white matter and meningeal membranes, and were chopped into 2-mm cubes. The cubes were lightly homogenized before incubation with collagenase. The digested material was dispersed by repeated aspiration through a pipette and, after partial purification by centrifugation through 25% bovine serum albumin, the recovered material was subjected to a second period of collagenase digestion. The microvessels were separated by adsorption onto columns of glass beads and purified by a series of washing and centrifugation techniques.

4) Preparation of Adipocytes and Myocytes. Adipocytes were prepared from the whole fat pad essentially by the procedure of Rodbell (J. Biol. Chem. 239: 375, 1974). Myocytes were obtained by the techniques of Lowe and Smallwood (Cancer Chemother. Pharmacol., in press). The latter procedure required perfusion of the cardiac vessels of heart initially with Ringer's solution (37°C) to remove blood (this perfusate was discarded) and then with Ringer's solution supplemented with collagenase at 37°C. This solution was recycled through the perfusion apparatus. The ventricles were then minced with scissors and subjected to six successive 15-min periods of digestion (37°C) with replacement of medium after each digestion. Myocytes were separated by centrifugation at 30 x g for 3 min and purified further by sedimentation through 3% v/v Ficoll at 60 x g.

5) Test for Cell Viability and Biochemical Assays. Aliquots of the microvessel and tissue cell suspensions were removed and tested for viability by the ethidium bromide diacetate staining procedure of Lionetti et al. (Transfusion 17: 465, 1977). Aliquots of myocyte suspensions were also transferred to a hemocytometer to determine the percentage of cells undergoing spontaneous beating. Tissue pellets and tissue specimens were stored at -20°C. For assay, they were suspended in Dulbecco's phosphate buffered solution (pH 6.4) and cells disrupted by sonification.

Histamine was assayed by the enzymatic isotopic assay, HMT activity by measurement of the rate of conversion of histamine to [^{14}C]methylhistamine in the presence of the labeled methyl donor, and DAO activity by measurement of tritium release from β-^{3}H-histamine. These procedures have been described in earlier reports. Protein was assayed by the procedure of Lowry et al. and DNA by a micromodification of the procedure of Hill and Whatley (FEBS Lett. 56: 20, 1975).

Major Findings: 1) Characteristics of Isolated Cell Preparations. The microvascular endothelial cell preparations were obtained with a high degree of purity and viability (95-100%). These consisted of single cells and segments of 2-8 cells, although those isolated from the brain cortex contained segments of greater length. Leukocytes and platelets were observed rarely (< 1%). Mast cells, myocytes, adipocytes, and neuronal elements were not observed in all preparations examined. Adipocyte and myocyte preparations showed a high degree of purity (∿ 95%). The myocytes were of lower viability (46-61%) than other cell preparations, although ∿ 30% (range, 17 to 50%) of the cells were spontaneously beating.

2) HMT Activity in Microvessel and other Cell Preparations. HMT activity in microvessels from guinea pig fat pads was approximately ten times that in whole tissue and > 25 times that in adipocytes. It was equally high in microvessels and myocytes from heart (on the basis of tissue weight and mg DNA). Because of the relatively severe conditions of digestion required to prepare isolated cells from myocardium, we could not be certain of complete recovery of HMT activity. The similarity of values in isolated myocytes and whole tissue (per mg DNA), however, suggested that losses, if any, were small.

HMT activity in microvessels from cerebral cortex was appreciable but was 1/4 that of the whole tissue. The activity in rat preparations was ony 1 to 7% that of the corresponding preparations from guinea pig, but the distribution and localization of HMT activity in microvessels was similar to that in guinea pig.

3) DAO and Histamine Levels in Microvascular Endothelial Cell Preparations. DAO activities were similar in all microvascular preparations with the exception of guinea pig heart microvessels. Although these activities were 4-50 times those in whole tissues, it was not as high as that in rat small intestine (5,400 ± 400 pmol/hr/g, n = 35) or guinea pig (n = 2) small intestine (2,200 pmol/hr/g), kidney (3,700 pmol/hr/g), and liver (9,400 pmol/hr/g). The cellular distribution of DAO has yet to be examined in these tissues. They were selected for reference as they represent the richest sources of DAO activity in these two species.

Histamine was present in all microvascular preparations from both species in amounts ranging from 0.1 to 0.6 μg/g tissue. Although significant, these levels were lower than those found in other body tissues.

4) Comparison of HMT Activity in Endothelial and other Cell Preparations. HMT activities in microvascular preparations from guinea pig fat pad and myocardium (4.3-4.4 units/hr/μg protein) exceeded those in all other cell and tissue preparations examined to date from this species (brain, 3.0 ± 0.1 units/hr/μg protein) and were slightly higher than that in dog parietal cells (3.4 units/hr/μg protein). These activities were also much higher than the isolated cell preparations examined from rats (< 0.1 units/hr/μg protein) but not as high as those in the two rat tissues, where HMT is highly localized (kidney and intestine, 37 and 7 units/hr/μg protein, respectively).

Significance to Biomedical Research: All microvascular endothelial preparations studied so far contain histamine-degrading activity and, in guinea pig, the levels of activity are particularly high. Such activity might account for the rapid inactivation of circulating plasma histamine and the almost complete removal of histamine upon perfusion across various vascular beds in several species. Since small increases (less than 5 ng/ml) in plasma histamine are associated with widespread urticarial and cardiovascular reactions in man and increases of 10-20 ng/ml are observed during severe anaphylactic shock, the presence of HMT and DAO activity at sites readily accessible to circulating histamine would be essential for survival, especially for humans in whom anaphylactic-type reactions are not uncommon (see Beaven, Anesthesiology 55: (July) 1981).. In addition to the vascular bed, two other histamine target cells with high HMT activity were identified, i.e., the guinea pig myocyte

and dog parietal cell. If further examples of an association of histamine-degrading activity and histamine receptors are found, a new dimension is added to the pharmacology of histamine receptors. A wide range of drugs (antihistamines, antimalarials, local anesthetics, histamine agonists) are relatively potent inhibitors of HMT and, in some cases, DAO activity (for example, see reference below), and some of these drugs have been shown to potentiate histamine's actions *in vivo* and *in vitro*.

Proposed Course: The localization of histamine-degrading enzymes in microvascular endothelial cells in tissues with high HMT and DAO activity, e.g., rat intestine and kidney, will be studied. In addition, the uptake and degradation of labeled histamine by intact endothelial cells will be compared with that of serotonin. Initial experiments will be carried out with microvessels from fat pad as the uptake and degradation of serotonin have been previously studied in this tissue by the principal investigator (J. Pharmacol. Exp. Ther. 216: 125, 1981).

Publications: Beaven, M.A. and Roderick, N.B.: Impromidine, a potent inhibitor of histamine methyltransferase (HMT) and diamine oxidase (DAO). Biochem. Pharmacol. 29: 2897-2900, 1980.

Almeida, A.P. and Beaven, M.A.: Phylogeny of histamine in vertebrate brain. Brain Res. 208: 1244-1250, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00632-01 CM

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Regulation of Hydroxymethyl CoA Reductase in Human Skin Fibroblasts

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

PI:	Joel Avigan	Research Chemist	CM	NHLBI
	Zafarul H. Beg	Research Chemist	MD	NHLBI

COOPERATING UNITS (if any)

Molecular Disease Branch, NHLBI.

LAB/BRANCH

Cellular Metabolism

SECTION

Metabolic Regulation

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.4	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)

Reversible phosphorylation of hydroxymethylglutaryl CoA (HMGCoA) reductase was shown for the first time in nonhepatic human cells in vitro. Phosphorylated and dephosphorylated (active) forms of the enzyme were assayed in human skin fibroblasts grown in culture. Short-term (30-60 min) incubation in medium containing lipoproteins of fibroblasts, that had previously been maintained in lipid-free medium, caused inactivation of the enzyme, which was reversed by the action of phosphatase. Longer incubation with lipoproteins caused irreversible inhibition of the enzyme. Mevalonolactone exerted an influence on HMGCoA reductase similar to that of serum lipoproteins.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: This project is an extension of Project No. Z01 HL 00623-02 CM and covers studies in the area of metabolic regulation of cholesterol synthesis in mammalian cells. We have earlier studied the enzyme HMGCoA reductase in cells grown in culture as part of investigations of cholesterol synthesis in mammalian tissues. Those studies, however, did not consider the possibility of rapid alterations of enzyme activity through phosphorylation and dephosphorylation. This regulatory mechanism has been demonstrated in vivo as well as in the hepatic microsomal preparations, but hitherto not in cultured cells. Demonstration of this mechanism in human skin fibroblasts contributes to the appreciation of the regulatory role of reversible phosphorylation in mammalian cells, in general, and in human ones, in particular, and it provides an opportunity to carry out studies on short-term physiological effects on the activity of the enzyme.

Methods Employed: Experiments are carried out with human skin fibroblasts grown in monolayers, having either depressed HMGCoA reductase activity after maintenance in medium containing whole serum or higher activity that results from incubation with lipid-free sserum. These cells are subsequently incubated for 15-240 min with media that affect the activity of the enzyme. The cells are washed, harvested, and frozen in a medium containing fluoride to inhibit the enzymatic hydrolysis of the phosphorylated form of HMGCoA reductase. Enzyme activity is then determined before and after enzymatic hydrolysis of the phosphate linkage.

Major Findings: It was demonstrated that rapid inactivation of HMGCoA reductase by serum lipoproteins in human fibroblasts was effected through a phosphorylation of the enzyme. Cells fully activated by long-term incubation with medium containing solvent-extracted serum contained no phosphorylated (inactive) HMGCoA reductase. Replacing this medium with one containing whole serum (low density lipoprotein) caused phosphorylation of the enzyme that peaked at approximately 1 hr with 35-38% being inactive unless dephosphorylated before assay. The depression of enzyme activity was due to phosphorylation, since it was totally reversed following the action of phosphatase. In cells incubated for 4 hr with serum lipoproteins, the inhibition was 93% of controls and no activation whatsoever occurred through the action of phosphatase. These observations indicate that, similarly to what was shown by others in animal liver, also in cultured cells, under prolonged influence of lipoproteins, the activity of HMGCoA reductase becomes more permanently depressed, perhaps due to reduction in enzyme protein either through reduced synthesis or increased degradation. Mevalonic acid, the first metabolic intermediate irreversibly committed to isoprenoid biosynthetic pathway, when added to incubation medium in the form of mevalonolactone, also initially, within 30 min, produced a phosphorylation of HMGCoA reductase completely reversed in the presence of phosphatase. At 60 min, the inhibition reached 46% and only 80% thereof could be restored by phosphatase.

Significance to Biomedical Research: Studies of the short-term regulation of enzymes producing cholesterol in cells will contribute to an understanding of this process in health and disease and provide a useful experimental

Proposed Course: The short-term effects of hormones known to influence the activity of HMGCoA reductase will be studied.

Publications: Mitchell, E.D., Jr. and Avigan, J.: Control of phosphorylation and decarboxylation of mevalonic acid and its metabolites in cultured human fibroblasts and in rat liver *in vivo*. J. Biol. Chem. 256: 6170-6173, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00633-01 CM

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Effect of Choleragen on Guanine Nucleotide Release from Turkey Erythrocyte Membranes

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

PI:	Drusilla L. Burns	Staff Fellow	CM	NHLBI
	Joel Moss	Head, Section on Molecular Mechanisms	CM	NHLBI
	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Cellular Metabolism

SECTION
Molecular Mechanisms

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.2	PROFESSIONAL: 1.2	OTHER: 0

CHECK APPROPRIATE BOX(ES)
☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
Choleragen has been shown to stimulate adenylate cyclase activity by catalyzing the ADP-ribosylation of a guanine nucleotide-binding regulatory protein (G/F). The effect of this chemical modification on the affinity of the G/F factor for guanine nucleotides was examined to determine the mechanism of choleragen action.

Treatment of turkey erythrocyte membranes with choleragen and NAD caused a stimulation in the release of guanine nucleotides from the membranes. β-Adrenergic agonists have been shown to stimulate adenylate cyclase by a mechanism in which the catecholamines induce release of GDP from the G/F factor. Guanine nucleotide-binding sites which are affected by the hormone agonists are identical to those which are altered by choleragen treatment, since pretreatment of the membranes with the toxin abolished subsequent stimulation of guanine nucleotide release by catecholamines.

Thus, choleragen and hormones may activate adenylate cyclase by similar mechanisms. Both types of stimulatory agents may act by decreasing the affinity of the G/F for GDP.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: Choleragen (cholera toxin) exerts its effects on cells by activating adenylate cyclase. The toxin acts by catalyzing the transfer of ADP-ribose from NAD to the guanine nucleotide-binding protein (G/F) of the adenylate cyclase complex.

Production of cAMP in the cell is dependent upon the binding of guanine nucleotides to the G/F. When GTP is bound at the regulatory site, the catalytic unit of the cyclase is activated. Hydrolysis of GTP to GDP is thought to terminate activation and result in a stable G/F-GDP complex. Hormones are believed to exert their effects on adenylate cyclase by promoting the exchange of bound GDP for GTP. Indeed, isoproterenol, a β-adrenergic agonist, has been shown to stimulate release of tightly bound [^{3}H]GDP from turkey ertyrocyte membranes coincident with activation of adenylate cyclase (Cassel, D. and Selinger, Z.: Proc. Natl. Acad. Sci. U.S.A. 75: 4155-4159, 1978).

Since choleragen modifies G/F, it was of interest to determine whether treatment of turkey erythrocyte membranes with the toxin and NAD alters the guanine nucleotide-binding properties of sites on the membranes, specifically those sites that are affected by isoproterenol and are thought to correspond to the G/F. In this manner, we hoped to determine whether stimulation of adenylate cyclase activity by choleragen is mediated through alterations in the guanine nucleotide-binding properties of the G/F.

Methods Employed: Binding of radiolabeled guanine nucleotides was initiated by addition of isoproterenol and [α-^{32}P]GTP to turkey erythrocyte membranes. The β-adrenergic agonist was included in this mixture in order to facilitate exchange of tightly bound guanine nucleotide for [α-^{32}P]GTP at hormone-sensitive sites. After a 2-min incubation, the membranes were washed extensively. Release of guanine nucleotides was initiated by the addition of choleragen and NAD or isoproterenol to the membranes and subsequently was assayed by the procedure described by Cassel and Selinger (Proc. Natl. Acad. Sci. U.S.A. 75: 4155-4159, 1978).

Major Findings: Exposure of turkey erythrocyte membranes to [α-^{32}P]GTP in the presence of isoproterenol followed by extensive washing of the membranes to remove the β-adrenergic agonist as well as loosely bound guanine nucleotides yields a preparation which is "loaded" with tightly bound guanine nucleotides. Subsequent incubation of this preparation at 37°C resulted in the release of radiolabeled guanine nucleotides into the incubation medium. When addition of choleragen was present, release during the course of one hour was increased by 40%. Choleragen-induced stimulation of guanine nucleotide release was dependent on the presence of NAD in the incubation medium, suggesting that the toxin enhances release by catalyzing the ADP-ribosylation of a membrane protein.

It appears that choleragen stimulates release of guanine nucleotides from the same sites that are affected by the isoproterenol. Isoproterenol-stimulated release was abolished by prior treatment of membranes with choleragen plus NAD for one hour. Thus, β-adrenergic hormones and choleragen may stimulate adenylate cyclase by similar mechanisms. That is, these agents may decrease the affinity of G/F factor for guanine nucleotides by increasing the

rate of release of GDP from the nucleotide-binding site, thus allowing the activating ligand, GTP, to bind.

Significance to Biomedical Research: Adenylate cyclase plays an important role in both heart and lung as well as numerous other tissues by mediating the actions of several hormones. Certain bacterial toxins including choleragen exert their effects on cells by activating this enzyme. This study has provided information which aids in understanding the molecular basis for activation of adenylate cyclase by the toxins and hormones.

Proposed Course: We hope to investigate further the regulation of adenylate cyclase by choleragen and hormones.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00634-01 CM

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Purification and Characterization of a Cyclic 3':5'-Guanosine Monophosphate-Stimulated Cyclic 3':5'-Adenosine Monophosphate Phosphodiesterase from Bovine Liver

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

PI:	Toshihiko Yamamoto	Visiting Fellow	CM	NHLBI
	Vincent C. Manganiello	Head, Section on Biochemical Physiology	CM	NHLBI
OTHER:	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Cellular Metabolism

SECTION
Biochemical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 2.2	PROFESSIONAL: 1.2	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)

A soluble low K_m form of <u>cyclic 3':5'-guanosine monophosphate phosphodiesterase (EC 3.4.1.17)</u>, which appears to be under <u>allosteric control</u>, has been partially purified from <u>bovine liver</u> homogenates. The preferred substrate for the enzyme is <u>cGMP</u> (K_m values: cGMP < cIMP < cAMP). The enzyme has a molecular weight of <u>360,000</u> daltons determined by gel filtration and a pI of 5.18 determined by iso-electric focusing. The <u>cAMP</u>-hydrolyzing activity is stimulated more than 20-fold by micromolar concentrations of cGMP. <u>p-(Hydroxymercuri)-benzoic acid</u> abolishes this positive cooperativity, presumably by oxidizing sulfhydryl groups on the enzyme essential for allosteric effects.

PHS-6040
(Rev. 2-81)

Project No. Z01 HL 00634-01 CM

Project Description:

Objectives: To elucidate the mechanisms through cyclic nucleotide phosphodiesterase (EC 3.4.1.17) activity is regulated in eukaryotic cells. Specifically, to purify a cyclic GMP-stimulated cAMP phosphodiesterase, to clarify the processes by which cyclic nucleotides induce conformational changes in the enzyme, and to study interrelationships (if any) between this enzyme and other phosphodiesterases.

Methods Employed: Phosphodiesterase is assayed by the procedure developed earlier in this laboratory. Progress on methodology for purification of the phosphodiesterase is described below.

Major Findings: Purification. The enzyme has been partially purified from bovine liver. Homogenates are centrifuged at 7,000 x *g* for 60 min and supernatants applied to DE 52 which is washed in a stepwise fashion with HEPES buffer (pH 7.2) containing increasing concentrations of NaCl. The cGMP-stimulated cAMP phosphodiesterase is eluted with 0.25 M NaCl and precipitated with $(NH_4)_2SO_4$. After dialysis, the proteins that remain in solution at pH 6.0 are applied to a column of DEAE-Sepharose and eluted in a gradient of NaCl. The enzyme is concentrated and applied to a column of Sepharose Cl-6B. These procedures both partially purify the cGMP-stimulated cAMP phosphodiesterase and separate this enzyme activity from other phosphodiesterases present in bovine liver homogenates. The enzyme is applied to columns of cAMP-Agarose and eluted with cAMP.

The enzyme is purified several thousandfold at this point and SDS gel electrophoresis indicates presence of predominantly 2 or 3 protein-staining bands of molecular weight in the range of 100,000 daltons.

Properties. At all stages of purification, there is a nonlinear relationship between enzyme activity and protein concentration. This enzyme hydrolyzes cGMP, cIMP, cAMP with the order of apparent K_m's: cGMP < cIMP < cAMP. In the presence of 1 μM [^{3}H]cAMP and 1 μM cGMP, activity is maximal in HEPES buffer, pH 7.2. cAMP hydrolysis is stimulated more than 20-fold by micromolar concentrations of cGMP. Low concentrations of *p*-(hydroxymercuri)-benzoic acid (PHMB) stimulate basal activity and reduce activation by cGMP. Higher concentrations of PHMB decrease both activities.

The enzyme behaves as an acidic protein with a pI of 5.18 determined by isoelectric focusing and a molecular weight of approximately 360,000 by gel filtration.

Significance to Biomedical Research: Several hormones and pharmacological agents exert their effects on the target cells by altering metabolism of intracellular cAMP and/or cGMP followed mainly by alteration of protein phosphorylation. Phosphodiesterases may play a significant role in terminating the signal produced by these effectors by hydrolyzing cyclic nucleotides. In addition, cGMP-stimulated cAMP phosphodiesterase considered here may be particularly important to maintenance of intracellular homeostasis by adjusting the cGMP/cAMP ratio.

Proposed Course: Continued purification to homogeneity and study of regulatory mechanisms of allosteric effectors.

Publications: Gagnon, C., Kelly, S., Manganiello, V., Vaughan, M., Odya, C., Strittmatter, W., Hoffman, A., and Hirata, F.: Enzymatic carboxyl methylation modifies calmodulin function. Nature, in press.

ANNUAL REPORT OF THE
LABORATORY OF CHEMICAL PHARMACOLOGY
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1980 - September 30, 1981

For nearly a decade this Laboratory has focused its efforts in discovering drugs and other foreign compounds that cause tissue lesions through the formation of chemically unstable metabolites. Among these compounds are halogenated benzenes, halogenated alkyl compounds, phenacetin, acetaminophen, isoniazid, iproniazid, several furyl compounds and spironolactone. The half-lives of the unstable metabolites vary widely but can be grossly classified. Ultra short-lived metabolites never leave the enzyme that catalyzes their formation; thus the initial damage is restricted to the enzyme. Short-lived metabolites (half-lives less than 1 sec) may cause cellular changes including cellular death, but the toxicities are restricted to cells in which the metabolites are formed. Long-lived metabolites (half-lives greater than 1 min) escape cells in which they are formed and are distributed throughout the body; thus virtually any cell in the body is potentially a target cell. Ultra long-lived metabolites (chemical half-lives of several hr) are frequently excreted into urine; thus much of the damage may occur in the bladder. Long-lived and ultra long-lived metabolites may frequently be identified directly. But the short-lived metabolites have been difficult to identify; indeed their structures are usually inferred from indirect evidence. During recent years, a major objective of the Laboratory has been to identify the metabolites and the enzymes that catalyze their formation and inactivation. Another objective has been to study the mechanisms by which the metabolites cause toxicities.

Formation and Decomposition of Chemcally Reactive Metabolites

Metabolism and toxicity of chloroform. We have previously reported evidence that rat liver microsomes convert chloroform ($CHCl_3$) to hydroxy trichloromethane (CCl_3OH), which spontaneously decomposes to hydrochloric acid and phosgene ($COCl_2$). In the presence of glutathione (GSH) the $COCl_2$ is converted to GS-CO-SG thereby leading to depletion of GSH in liver. The hepatotoxicity of chloroform is presumably caused by CCl_3OH or $COCl_2$ because $CDCl_3$, which is converted to $COCl_2$ less rapidly than is $CHCl_3$ is also less toxic. The treatment of rats with methyl n-butyl ketone (MBK) or other aliphatic secondary alcohols and ketones markedly potentiates the toxicity of chloroform in liver and kidney, but the mechanism has been obscure. During the past year, we obtained evidence that MBK evokes its action in at least two ways: 1) MBK causes a decrease in GSH concentrations in liver and 2) the treatment accelerates the metabolism of $CHCl_3$ and thus speeds the depletion of GSH concentrations in liver and the appearance of GS-CO-SG in bile. The accelerated conversion of $CHCl_3$ to GS-CO-SG caused by treatment with the ketone is apparently due to an increase in microsomes of a form of cytochrome P-450 having a mole wt of 72,000. The potentiation of the renal toxicity of $CHCl_3$ caused by prior treatment of rats with MBK remains obscure. Although MBK does not increase renal cytochrome P-450, the finding that $CDCl_3$ is less toxic than $CHCl_3$ in kidneys suggests that the toxicity requires metabolism of chloroform.

Metabolism and toxicity of carbon tetrachloride. In the absence of oxygen, liver microsomes convert carbon tetrachloride (CCl_4) to $CHCl_3$ presumably via the trichloromethyl free radical ($Cl_3C\bullet$). In the presence of oxygen, however, CCl_4 is converted to $COCl_2$ but less rapidly than is $CHCl_3$. Previously it was

thought that CCl_4 is first converted to $CHCl_3$ and then to $COCl_2$, but it is now evident that $COCl_2$ is formed from $CHCl_3$ and CCl_4 by different mechanisms.

The realization that the hepatotoxicity of CCl_4 cannot be caused solely by $COCl_2$ prompted a search for other chemically reactive metabolites of CCl_4. We have discovered that rat liver microsomes under air convert CCl_4 to a metabolite that reacts with 2,6-dimethyl phenol to form 4-chloro-2,6-dimethyl phenol. Under these conditions, $CBrCl_3$ also leads to the formation of 4-chloro-2,6-dimethyl phenol, but CBr_4 leads to the formation of 4-bromo-2,6-dimethyl phenol. These findings suggest to us that CCl_4 and $CBrCl_3$ are reductively cleaved to $Cl_3C\bullet$ which reacts with oxygen to form a trichloromethyl superoxide ($Cl_3CO_2\bullet$). The $Cl_3CO_2\bullet$ then decomposes either directly or indirectly to $COCl_2$ and an electrophilic chlorine (Cl_2 or OCl^-), perhaps via trichloromethyl peroxide.

Chloramphenicol metabolism. During the past several years our studies of the metabolism of chloramphenicol have revealed several unusual reactions catalyzed by different hepatic enzymes. Last year we reported that in the presence of air, a cytochrome P-450 in liver microsomes catalyzes the cleavage of chloramphenicol to p-nitrobenzyl alcohol. Since then we have identified the other cleavage product of chloramphenicol as N-(2-hydroxyethyl) dichloroacetamide ($HOCH_2CH_2NHCOCHCl_2$). The reaction apparently is catalyzed only by a form of cytochrome P-450 that is induced during the treatment of rats with phenobarbital. Neither product is formed by liver microsomes from untreated rats or from rats treated with β-naphthoflavone.

Last year, we also reported that in the absence of oxygen, a form of cytochrome P-450 in rat liver microsomes catalyzed the formation of monodeschlorochloramphenicol by a reaction presumably analogous to the reductive cleavage of CCl_4 to $Cl_3C\bullet$ and thence to $CHCl_3$. In accord with this view, chloramphenicol labeled with ^3H and ^{14}C in different positions was covalently bound to protein when incubated with liver microsomes in the absence of oxygen. The reaction is apparently catalyzed by a form of cytochrome P-450 induced during the treatment of rats with phenobarbital; very little if any monodeschlorochloramphenicol or chemically reactive metabolite was formed under nitrogen when chloramphenicol was incubated with liver microsomes from untreated rats or rats treated with β-naphthoflavone.

Surprisingly, chloramphenicol is not converted to chloramphenicol amine when the drug is incubated with liver microsomes in the absence of oxygen. Thus, the identity of the metabolite that is known to react with the Bratton and Marshall reagent remains obscure. Nevertheless, the intestinal flora under nitrogen does reduce chloramphenicol to chloramphenicol amine. The formation of the amine presumably occurs through the formation of nitrosochloramphenicol, a substance that is highly toxic to bone marrow cells *in vitro* but does not cause aplastic anemia when administered to mice or calves. We have found that nitrosochloramphenicol rapidly reacts with GSH to form a GSH conjugate that decomposes to the hydroxylamine analogue of chloramphenicol. Neither the GSH conjugate nor the hydroxylamine analogue is toxic to cultures of bone marrow cells.

Acetaminophen metabolism. The chemically reactive metabolite of acetaminophen formed by liver microsomes is known to react with GSH to form a GSH conjugate, but the position at which the GSH is attached to the acetaminophen molecule

has been uncertain. In collaboration with Dr. Robert Highet (Lab. of Chemistry, NHLBI), we have established unequivocally that the conjugate is 3-(glutathion-S-yl) acetaminophen.

Bromobenzene metabolism. Nearly a decade ago, our Laboratory established that bromobenzene causes liver necrosis through the formation of an epoxide, which reacts with GSH to form a conjugate that is ultimately excreted into urine as a mercapturate. The toxic epoxide appeared to be bromobenzene-3,4-oxide, because treatment of animals with phenobarbital, which increased the toxicity, also increased the formation of p-bromophenol, another decomposition product of the epoxide, whereas treatment of animals with 3-methylcholanthrene, which decreased the toxicity, increased the formation of o-bromophenol.

The position at which the GSH reacted with the epoxide has been uncertain. During the past year, we have isolated two GSH conjugates from the bile of rats receiving bromobenzene and in collaboration with Dr. Robert Highet (Lab. of Chemistry, NHLBI) have confirmed their structure by ^{13}C and ^{1}H NMR. The predominant form, representing about 63% of the total, was identified as trans-3-bromo-6-(glutathion-S-yl)-cyclohexa-2,4-diene-1-ol. The other form, was identified as trans-4-bromo-6-(glutathion-S-yl)-cyclohexa-2,4-diene-1-ol. The relative amounts of the two forms is surprisingly constant, the ratio is not changed by changing the size of the dose or by pretreatment of rats with either phenobarbital or 3-methylcholanthrene. Our finding thus suggests that both isomers are formed from bromobenzene-3,4-oxide, perhaps by a single transferase in liver.

The formation o-bromophenol is thought to occur via either bromobenzene-1, 2-oxide or bromobenzene-2,3-oxide. However, we have been unable to detect a GSH conjugate that could be attributed to either of these arene oxides.

Pharmacokinetic Studies

Differentiation between short-lived and long-lived chemically reactive metabolites. One of the key objectives in any study of the toxicity of chemically reactive metabolites should be to determine whether the toxicity will be restricted to those cells in which the metabolite is formed or whether virtually every cell in the body must be considered as a potential target cell. To this end we are developing techniques to determine whether chemically reactive metabolites escape cells in which they are formed. In our basic approach, precursors of the chemically reactive metabolites are incubated with hepatocytes with and without proteins that trap chemically reactive metabolites, or with and without enzymes that catalyze the inactivation of the chemically reactive metabolites. With this approach, we have obtained evidence that bromobenzene-3,4-oxide leaves hepatocytes.

Pharmacokintics of imipramine and its metabolites. A long term objective of the Laboratory has been to evaluate problems in extrapolating data obtained from *in vitro* studies to living animals. Studies of the conversion of imipramine to its demethylated and hydroxylated metabolites illustrate some of these problems. Studies with liver microsomes from rats indicate that at low concentrations of imipramine (I), the rates of formation of desipramine (DMI) and hydroxyimipramine (HOI) should be about the same. At high concentrations of I however, the formation of DMI should be significantly greater than the formation HOI. Studies with liver microsomes also indicate that in the absence of cofac-

tors required for the glucuronidation of HOI, the rate of formation of hydroxydesipramine (HODMI) should initially be faster from HOI than from DMI, although ultimately the relative importance of the two sources of HODMI should reflect the relative rates of formation of DMI and HOI. Hepatocytes convert HOI to its glucuronide and thus the importance of this intermediate in the formation of HODMI is diminished but still measurable. *In vivo*, however, the rate formation of glucuronide of HOI is so efficient that the amount of HODMI formed from HOI is negligible. Thus *in vivo*, virtually all of the HODMI is formed from DMI. As previously pointed out, however, DMI forms an enzymatically inactive complex with microsomal cytochrome P-450, which is slowly reversible. Thus the complex leads to the inhibition of I and DMI metabolism. Since the formation of the complex can be blocked by high concentrations of I or DMI the inhibition is manifested only when the concentrations of the two substrates are low. Thus, the intrinsic clearance of the enzyme that converts DMI to HODMI appears to be greater at high doses than at low doses of I.

Pharmacokinetic methods for estimating hepatic blood flow rates. As pointed out in previous reports, when a drug is eliminated by both the kidney and the liver, the ratio of the areas under the curves of the blood concentration of a metabolite formed solely in the liver after the drug is administered both orally and intravenously should be $(1+Cl_R/Q_H)$, where Cl_R is the renal clearance of the drug and Q_H is the hepatic blood flow rate. When a precursor is also converted to the drug solely by the liver, intravenous administration of the precursor may replace the oral administration of the drug. Ideally, the precursor should be eliminated from the body solely by way of the drug, but a modification of our equations reveals that this is not absolutely necessary. Although the modified approach broadens our consideration of possible precursor-drug-metabolite combinations, we still have not found a suitable combination.

Pharmacokinetics of cephaloridine excretion. The renal clearances of cephaloridine and cephapirin in humans decrease as their concentrations in blood decrease, suggesting the presence of an active transport system in kidney tubules that mediates the reabsorption of the drugs (Arvidsson *et al*. Clin. Pharmacol.Therap.25:870,1979). Last year we reported evidence that the normal role of the reabsorption transport system might be the reabsorption of uric acid. Evidence obtained during the past year, however, indicates that this is unlikely. The normal role of the system, thus remains unknown.

Multiforms of Cytochrome P-450

It has become increasingly evident that liver microsomes contain several different forms of cytochrome P-450. Indeed it has been estimated that all the forms which have been purified and characterized account for only about 15% of the cytochrome P-450 in liver microsomes of untreated rats. Several approaches have been used by the Laboratory to identify the various forms.

Induction by hydrazine. Last year we confirmed the findings of Rice *et al*. that isoniazid and hydrazine in rats markedly increase the rate of metabolism of enflurane by liver microsomes. We now report that even though the treatment with hydrazine does not increase the total amount of cytochrome P-450 in liver microsomes, it increases the amount of a minor form of cytochrome P-450. Unfortunately the form is unusually unstable in the solubilized state and rapidly changes to cytochrome P-420. Treatment with hydrazine in-

creases the metabolism of enflurane by liver microsomes from rats, hamsters, mice and guinea pigs but apparently not those from rabbits.

Metabolism of B-adrenergic blockers. Last year, we reported that treatment of rats with phenobarbital increases the conversion of oxprenolol to desisopropyloxprenolol and desallyloxprenolol, by liver microsomes but blocks its conversion to 4-hydroxyoxprenolol. By contrast treatment of rats with B-naphthoflavone increases the metabolism of oxprenolol by all three reactions. We also reported the R-form of the drug was converted to 4-hydroxy oxprenolol more rapidly than was the S-form, whereas the S-form was converted to desallyoxprenolol more rapidly than was the R-form. These findings raised the possibility that the different reactions by which oxprenolol is metabolized are catalyzed by different forms of cytochrome P-450.

During the past year W. Levin (Hoffmann-LaRoche) provided us with antibodies against cytochrome $P\text{-}450_b$, the predominant form induced by treatment of rats with phenobarbital, and against cytochrome $P\text{-}450_c$, the predominant form induced by treatment of rats with either 3,4-benzpyrene or β-naphthoflavone. With these antibodies we have found that cytochrome $P\text{-}450_b$ does not catalyze the metabolism of oxprenolol; instead phenobarbital must induce the synthesis of another form that catalyzes the metabolism of oxprenolol to desisopropyloxprenolol and desallyloxprenolol. Although cytochrome $P\text{-}450_c$ catalyzes the metabolism of oxprenolol to all three metabolites it is not the only form of cytochrome P-450 that metabolizes the drug; indeed in microsomes from untreated rats it accounts for very little of the metabolism of the drug.

Like oxprenolol propranolol also contains a 1-isopropylamino-2-propanol group and thus an asymmetric carbon atom. Propranolol is metabolized by rat liver microsomes to: 7-hydroxypropranolol, 4-hydroxypropranol and desisopropylpropranolol. Studies with liver microsomes from rats treated with either phenobarbital or B-naphthoflavone revealed that cytochrome $P\text{-}450_c$ probably catalyzes the formation of desisopropylpropranolol and 4-hydroxypropranolol but does not catalyze the formation of 7-hydroxypropranolol. Cytochrome $P\text{-}450_b$ also does not catalyze the formation of 7-hydroxypropranolol but whether cytochrome $P\text{-}450_b$ or other forms of cytochrome P-450 catalyzes the other two reactions remains to be determined.

Mechanisms of Toxicity and Drug Action

Effects of Ca^{++} on toxicity in hepatocytes. We have used recently developed improvements in the conditions for primary cultures of hepatocytes which maintain cytochrome P-450 concentrations and the activities of various drug metabolizing enzymes for at least 24 hr. With these improved conditions we have studied the effects of Ca^{++} in the culture medium on the cellular damage caused by various toxicants and have discovered that 10 uM Ca^{++} diminishes cytotoxicity but 3.6 mM Ca^{++} potentiates the action of some but not all toxicants.

Ordinarily the unsupplemented medium contains 10-20 uM Ca^{++}. At this concentration of Ca^{++}, the ionophore A23187 is relatively nontoxic. However, the toxicity may be greatly enhanced either by depleting the medium of unbound calcium by the addition of a chelating agent, EGTA, or by addition of physiological concentrations of Ca^{++} (3.6 mM). Scanning electron microscopy (SEM)

revealed that the ionophore in the presence of 3.6 mM Ca^{++} causes a rounding of the hepatocytes, loss of microvilli and the appearance of numerous blebs and balloons, which are continuous with the plasma membrane. Transmission electron microscopy revealed that the blebs contain a clear matrix, largely devoid of cytoplasmic organelles, but that the major organelles in cells are unaffected, other than swelling of the mitochondria. Since uncouplers of oxidative phosphorylation often cause swollen mitochondria, it at first seemed possible that depletion of ATP might account for the toxic effects of the ionophore. In support of this view, 10^{-4}M dinitrophenol, which decreases the concentration of ATP by 99%, also caused cytotoxicity; the cytotoxicity is potentiated by addition of either ethionine, which depletes ATP through the formation of S-adenosyl ethionine, or the ionophore. But it seems unlikely that the toxicity caused by the ionophore is due solely to depletion of ATP. Although the ionophore decreases the concentration of ATP by 60%, the addition of ethionine does not potentiate the toxicity.

Last year we reported that 3.6 mM Ca^{++} potentiated the cytotoxicity of CCl_4, but during the past year we discovered that the addition of EGTA to the medium also potentiates it. By contast the cytotoxicity caused by acetaminophen is also increased by EGTA, but is not affected by the addition of 3.6 mM Ca^{++}. It is evident, therefore, that the manifestation of cytotoxic effects caused by acetaminophen does not require the transfer of extracellular Ca^{++} into cells. Whether intracellular Ca^{++} plays a role in toxicity remains to be determined.

Mechanisms of prostacyclin action. Prostacyclin (PGI_2) inhibits platelet aggregation by activating adenylate cyclase and thereby increasing cyclic AMP synthesis. During the past year we discovered that GTP is required for the maximal activation of adenylate cyclase not only by PGI_2 but also by several active analogues, such as 20-methyl PGI_2, 13,14-didehydro-20-methyl PGI_2 and 13,14-didehydro-16-methyl-PGI_2. However, inactive analogues, such as 20-normethyl PGI_2 and 8,9-dimethyl-9-methylene-15-epi PGI_2, fail to activate adenylate cyclase even in the presence of GTP.

Human platelets possess receptors for PGI_2 as determined by the specific binding of ^3H-PGI_2. However, there are marked differences in the number of receptors among individuals. The finding that the number of receptors appear to correlate with the effectiveness of PGI_2 in activating adenylate cyclase and in inhibiting platelet aggregation may aid in identifying individuals at risk in the development of cardiovascular diseases.

Antibodies against guanylate cyclase. We are continuing our efforts to develop monoclonal antibodies against guanylate cyclase by hybridoma techniques. So far, we have found four colonies that produce small amounts of monoclonal antibodies against the enzyme.

Technical Developments

Development of flow cell-microprocessor units. The current techniques for purification of cytochrome P-450 enzymes by column chromatography require time consuming spectrophotometric assays of these hemoproteins. We are currently building a prototype of an assay system in which cytochrome P-450 enzymes may be measured instantaneously as they are eluted from columns.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00810-04 LCP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Kinetics of formation and elimination of drug metabolites by the liver

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

P.I.:	Terrence J. Monks	Vist. Fellow	LCP	NHLBI
Other:	James R. Gillette	Chief	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme-Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Last year we reported the development of a sensitive HPLC assay for imipramine and its metabolites. With this method we have studied the kinetics of imipramine metabolism in isolated rat liver hepatocytes and compared the results to those obtained in rat liver microsomes and in vivo. The results indicate that whilst isolated rat liver hepatocytes offer a better in vitro model of imipramine metabolism than do rat liver microsomes, they do not completely mimic the in vivo situation.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: Previous studies indicated the formation of an inactive cytochrome P-450 complex when NADPH is added to microsomal incubations in the presence of desipramine. However, the complex does not form in the presence of large concentrations of desipamine and imipramine. The purpose of the present study was to determine whether a similar complex forms during the metabolism of imipramine in isolated rat liver hepatocytes and whether this complex inhibits imipramine metabolism. The application of isolated rat liver hepatocytes as a better *in vitro* model for drug metabolism will also be determined.

Methods Employed: Isolated hepatocytes have been prepared by a two-step collagenase perfusion technique. Initially interstitial calcium is removed by the chelator EGTA followed by perfusion with medium containing 5 mM calcium chloride and 0.05% collagenase for about 10 min. Isolated cells are then suspended in a complete nutrient medium and investigated for their ability to metabolize imipramine.

The kinetics of ^{14}C imipramine metabolism were studied in control suspensions and also in suspensions preincubated with various concentrations at desipramine prior to the addition of ^{14}C imipramine.

To clarify the probable *in vivo* source of hydroxydesipramine, the fate of an i.v. dose of hydroxyimipramine was investigated in bile duct cannulated rats.

Major Findings: The effect of preincubation with desipramine on ^{14}C-imipramine in isolated hepatocytes is not as clearly defined as expected. This is probably due to the less rapid and less extensive formation of a desipramine cytochrome P-450 product complex in hepatocytes than in microsomes. This is in accord with other workers (Hirata *et al.*, 1977) who showed a similar finding with amphetamine derivatives. Furthermore, optimum conditions for the formation of the desipramine cytochrome P-450 product complex have not been established in hepatocytes as yet. Thus whilst preincubation of hepatocytes with desipramine concentrations between 10-20 uM impair imipramine metabolism, the degree of inhibition observed is not significant.

Hydroxydesipramine can be formed via demethylation of hydroxyimipramine or by hydroxylation of desipramine. We have previously shown that both pathways exist in rat liver microsomes and that this *in vitro* system predicts that at the concentrations present *in vivo* about 50% of the hydroxydesipramine would be formed via hydroxyimipramine. However, there is a rapid "first-pass" removal of hydroxyimipramine from the rat *in vivo*, with large amounts of the corresponding glucuronide conjugate being excreted into bile. Furthermore, no hydroxydesipramine could be detected in blood, bile or urine following i.v. hydroxyimipramine. Such a rapid removal of hydroxyimipramine precludes it as being the major *in vivo* source of hydroxydesipramine in rats.

Results with rat liver hepatocytes suggest that the rapid *in vivo* conjugating systems are not as efficient in isolated hepatocyte suspensions. The inability to remove any hydroxyimipramine glucuronide from the incubation medium renders it susceptible to hydrolysis by B-glucuronidase activity present in the suspension. Thus neither of the two *in vitro* systems accurately predicts the *in vivo* situation although isolated hepatocyte suspensions do offer the better *in vitro* alternative.

Significance to Biomedical Research and the Program of the Institute: The extent of demethylation of imipramine may be clinically important since the metabolite thus formed is pharmacologically active. It is, therefore, important to recognize any factor(s) influencing the formation of this metabolite and to elucidate the mechanisms responsible.

Proposed Course of Project: We have shown that isolated rat liver hepatocytes have the potential to form the cytochrome P-450 complex observed in microsomes incubated with NADPH and desipramine. We intend to investigate the conditions necessary for the optimum formation of this complex in isolated hepatocytes and thus to better identify its effects on imipramine metabolism.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00818-01 LCP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Regioselectivity in propranolol metabolism by rat liver microsomes

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

P.I.:	Henry A. Sasame	Chemist	LCP	NHLBI
Others:	None			

COOPERATING UNITS (if any)

Dr. Wendel Nelson, University of Washington, Seattle, Washington.

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Enzyme-Drug Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

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)

The relative rates of formation of three major metabolites from the R and S isomers of propranolol, a widely used B-adrenergic blocker, varies with the metabolite and the treatment of rats. 7-Hydroxypropranolol was formed by liver microsomes from untreated rats but not by those from rats pretreated with phenobarbital or B-naphthoflavone. The relative rate of formation of 7-hydroxypropranolol (R/S) ranged from 1.8-2.1. By contrast the R/S ratios for the formation of 4-hydroxypropranolol and desisopropylpropranolol by liver microsomes from either untreated rats or rats treated with phenobarbital were 1.0. With microsomes from B-naphthoflavone treated rats, however, the R/S values ranged from 1.45-1.82 for 4-hydroxypropranolol and from 1.6-2.0 for desisopropranolol formation.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: Last year we had presented evidence for regioselectivity in the metabolism of oxprenolol by rat liver microsomes which depended upon the type of pretreatment of the rats and the metabolite. Like oxprenolol propranolol has an isopropyl group and thus an asymmetric carbon. It thus seemed likely that the metabolism of propranolol by rat liver microsomes would also be regioselective. Since propranolol is a widely used antihypertensive drug, it seemed pertinent to investigate this regioselectivity.

Methods Employed: The experimental techniques were essentially the same as those used in the study of oxprenolol metabolism.

Major Findings: 1) The formation of 7-hydroxypropranolol (7-OHP) was detected with liver microsomes from untreated rats but not with those from rats treated with phenobarbital or BNF. 2) The relative rates of formation of 7-OHP from the S and R isomers (R/S) ranged from 1.9-2.1. By contrast, the R/S values for the formation of 4-hydroxypropranolol (4-OHP) and desisopropylpropranolol (DIP) by liver microsomes from either untreated rats or rats pretreated with phenobarbital were 1.0. With liver microsomes from BNF treated rats, the R/S values ranged from 1.45-1.82 for 4-OHP formation and from 1.66-2.05 for DIP formation. 3) Both phenobarbital and B-naphthoflavone increased the rates of formation of both 4-OHP and DIP formation per mg microsomal protein. 4) The relative rates of formation of either 4-OHP or DIP from the R and S isomers by microsomes from animals treated with BNF remained constant as the pH was varied from pH 6.5 to 8.0 suggesting that pH has little effect on regioselectivity of the forms of cytochrome P-450 with the stereoisomers of propranolol.

Significance of Biomecial Research and the Program of the Institute: A better understanding of regioselective metabolism of propranolol should provide a better understanding of the enzymes that metabolize propranolol in rats.

Proposed Course of Project: The kinetic analysis of propranolol metabolism from S and R isomers should be carried out with purified cytochrome P-450 enzyme isolated from liver microsomes from BNF induced rats. In addition, immunochemical studies with antibodies against specific forms of cytochrome P-450 should provide a further understanding of propranolol interaction with cytochrome P-450.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00845-02 LCP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Guanylate cyclase: preparation of monoclonal and polyclonal antibodies

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

P.I.	Gopal Krishna	Chief, Section	LCP	NHLBI
Others:	Thomas Hundley	Bio. Lab. Tech.	LCP	NHLBI
	Nancy Kim	Chemist	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Drug-Tissue Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS: 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)
A simple radioimmunoassay has been developed utilizing guanylate cyclase isolated from sea urchin sperms. The enzyme was purified as reported last year to single electrophoretic band purity and was iodinated with [^{125}I] Bolton-Hunter reagent. The antigen antibody complex was precipitated with anti IgG. The assay was capable of measuring subpicomoles of guanylate cyclase or antibody. This method has been used to detect antiguanylate cyclase antibody production in myeloma hybridoma cell cultures.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: One of the main objectives of this study is to develop a simple radioimmunoassay that could be used for the quantitation of both guanylate cyclase and specific antibodies produced against this enzyme. Another objective is to use the assay for screening hybridoma cells that produce specific antibodies against guanylate cyclase.

Methods Employed: Guanylate cyclase was purified as reported earlier. The enzyme was electrophoretically pure and had an apparent molecular weight of 160,000. The enzyme was iodinated by reaction with [^{125}I] Bolton-Hunter reagent and purified by chromatography. For the assay, [^{125}I] antigen and antibody were incubated and the antigen-antibody complex was precipitated by a second specific anti IgG.

Major Findings: The assay was capable of detecting subpicomole quantities of either guanylate cyclase or specific antibodies. The antibody production in animals such as rabbit and mouse has been followed by this assay. Spleen cells isolated from mice that produce antibodies against guanylate cyclase were fused with mouse myeloma cells and the hybridomas were selectively isolated and cloned. Various colonies were subcultured over a period of 4-6 weeks and the medium was routinely monitored for antibody production. So far 4 colonies have been identified as producers of antiguanylate cyclase antibodies. These cells appear to produce the antibodies in small amounts and may require further subcloning in order to isolate the cells that are prolific in producing specific antibodies against guanylate cyclase.

Significance to Biomedical Research and Program of the Institute: The experience gained in the production of monoclonal antibody will be useful for production of other specific antibodies directed against various forms of cytochrome P-450.

Proposed Course of Project: We propose to use the specific antibody against guanylate cyclase to localize the enzyme in various tissues in order to understand the role of cyclic GMP in various functions. We also propose to use hybridomas for production of monoclonal antibodies directed against the various forms of cytochrome P-450.

Publications:

Chader, G.J., Fletcher, R.T. and Krishna, G.: Guanine nucleotides: Impotance in visual processes of the rod outer segment. New Directions in Ophalmic Research, in press.

Gaion, R. and Krishna, G.: A possible role of calcium and cyclic GMP in hormone-induced lipolysis. in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00846-02 LCP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Effect of chemical modification of prostacyclin on its action

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

P.I.:	Mariam George	Vist. Fellow	LCP	NHLBI
	Gopal Krishna	Chief, Section	LCP	NHLBI
Other:	Nancy Kim	Chemist	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Drug-Tissue Interaction

INSTITUTE AND LOCATION

NHBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	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)

Various PGI_2 analogues and PGI_2 were shown to activate maximally platelet membrane adenylate cyclase only in the presence of GTP. The inactive analogues of PGI_2 did not activate adenylate cyclase even in the presence of GTP, indicating the analogues may have less avidly bound to PGI_2 receptors.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: The prostaglandins are a group of complex lipids which have been shown to produce a variety of effects in biological systems. Prostacyclin (PGI_2) is a potent inhibitor of platelet aggregation. It increases the synthesis of cAMP by activating platelet adenylate cyclase. Although PGI_2 plays an important physiological role in platelet function, studies of its effects have been limited because it is unstable at physiological pH.

The main purpose of the study is to investigate the effects of various PGI_2 analogs on platelet adenylate cyclase and GTP on the PGI_2 activate platelet membrane adenylate cyclase. We have shown earlier that GTP is absolutely required for the activation of platelet adenylate cyclase by PGE_1.

Methods Employed: Purified platelet membranes were prepared from human blood platlets. PGI_2 activated adenylate cyclase was assayed in the membrane was assayed in the presence or absence of GTP.

Major Findings: Using the purified platelet membrane fraction we studied the GTP requirement of adenylate cyclase activity. The preliminary experiments showed that without GTP, the PGI_2 did not maximally activate the adenylate cyclase. In the absence of GTP, the PGI_2 analogs also did not activate the adenylate cyclase. In the presence of GTP, however, PGI_2 and some of the analogs (13,14-didehydro-20-methyl PGI_2, 20-methyl PGI_2, and 13,14-didehydro-16-methyl PGI_2) showed marked activation of adenylate cyclase, while others (20-nor methyl PGI_2, and 8,9-dimethyl-9-methylene-15 epi PGI_2) showed very little activation even in the presence of GTP. The activation of adenylate cyclase by various PGI_2 analogs was similar to that reported last year on cAMP levels.

These experiments demonstrate a requirement for GTP in the PGI_2 dependent activation of adenylate cyclase in the purified plasma membrane from human platelets. The inactive analogs were inactive even in the presence of GTP indicating that the defectiveness is probably not due to differences in the GPT requirement, instead it may be due to differences in their affinity to the PGI_2 receptor or to the formation of inactive complexes.

Significance to Biomedical Research and Program of the Institute: PGI_2 and its analogs are currently under intensive study for use in the treatment of a wide variety of disorders, including myocardial infraction, angina, vascular occulsive diseases and hypertension. Knowledge of the requirement of GTP in PGI_2 activation of adenylate cyclase may aid in the understanding the lack of PGI_2 response in certain individuals.

Proposed Course of Project: We propose to study PGI_2 receptors in blood platelets and to compare the efficacy of the analogs to bind to the receptor and to displace the bound PGI_2. We also propose to investigate

why certain analogs were more active in some individuals than in others and to see if they have any inhibiting effect in the system.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00847-02 LCP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Role of calcium in CCl_4-induced hepatotoxicity

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

P.I.:	Richard Chenery	Vist. Fellow	LCP	NHLBI
	Mariam George	Vist. Fellow	LCP	NHLBI
	Gopal Krishna	Chief, Section	LCP	NHLBI

Other: None

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Drug-Tissue Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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)

In our last report we described the potentiation of CCl_4-induced damage by calcium in cultured hepatocytes. Potentiation of cell injury was only observed at levels of CCl_4 which caused moderate cell damage, indicating that calcium uptake across the plasma membrane may not be obligatory for the development of cell injury. We have therefore clarified the role of external calcium in the development of xenobiotic induced cell injury by utilizing three compounds with different primary modes of toxicity; i.e., acetaminophen, 2,4-dinitrophenol and ionophore A23187. Addition of 100 uM EGTA to culture medium without added calcium completely removes free calcium Incubation of cultured hepatocytes from adult rats induced with phenobarbital in vivo in this calcium free medium with 8 mM acetaminophen resulted in extensive cell damage. The damage was greater at pH 7.6 than at pH 7.2. Addition of physiological concentrations of calcium after 2 hr incubation resulted in a dramatic decrease in apparent toxicity. These changes appeared to be unrelated to alterations in acetaminophen metabolism but rather to changes in sensitivity to cell damage.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: We have shown that physiological levels of calcium in the culture medium potentiate the cell damage induced by CCl_4, relative to culture medium containing no added calcium (10-14 uM calcium). In these experiments calcium has been reduced to less than 1 uM by the addition of 100 uM EGTA. This system allows critical assessment of the role of extracellular calcium in xenobiotic-induced cellular damage.

Methods Employed: Isolated hepatocytes were prepared as described in the last report. Cells were either plated out in poly-L-lysine coated Falcon flasks and used after 1 hr of culture or on to collagen coated petri dishes and cultured 24 hr before use. 2,4-Dinitrophenol and acetaminophen were dissolved directly in culture medium whilst either ethanol or DMSO were used as solvent for the ionophore A23187.

Metabolism of acetaminophen was studied by measuring the appearance of glucuronide, sulfate or glutathione conjugates in the culture medium by HPLC. Glutathione was measured by the ortho phthalaldehyde fluorometric technique. Cellular damage was assessed by measuring leakage of cytoplasmic enzymes into the culture medium and by changes in cell morphology.

Calcium concentrations in the culture medium were estimated with the dye Arsenazo III.

Major Findings. Cultured hepatocytes extensively metabolized acetaminophen to glucuronide, sulfate and glutathione conjugates. At 8 mM acetaminophen, glucuronidation and sulfation were effectively saturated; increasing the concentration of the analgesic to 25 mM actually decreased the production of these two conjugates. However, production of glutathione conjugate was much greater with 25 mM than with 8 mM acetaminophen. This was associated with decreased cellular glutathione levels and the development of significant enzyme leakage.

When cells were incubated with 8 mM acetaminophen in the absence of calcium (100 uM EGTA) enzyme leakage occurred within 2 hr, and was highly dependent upon the pH of the culture medium;leakage was much greater at pH 7.6 than pH 7.2. Addition of physiological calcium concentrations to these cultures after 2 hr incubation afforded considerable protection against cell injury compared to calcium-free medium. Thus, entry of external calcium into the cell across the plasma membrane was not an obligatory step in the development of cell injury. The protective role of calcium suggests that low levels of calcium may be required to maintain plasma membrane function under conditions of mild injury.

We have previously shown that the covalent binding of CCl_4 was insensitive to calcium levels in the culture medium. Similarly, the production of conjugates of acetaminophen are apparently unchanged upon the removal of calcium from the culture medium. Thus the large increase in toxicity observed upon calcium removal cannot be explained by an altered metabolism.

2,4-Dinitrophenol (DNP) is a well-known uncoupler of oxidative phosphorylation which induced enzyme leakage from culture hepatocytes at about 10^{-4}M. The injury induced by DNP was extensive when calcium was totally removed from the culture medium and was also pH dependent, being greater at pH 7.6 than 7.2. Similar results were obtained with the ionophore A23187. This compound was relatively nontoxic at low calcium concentrations (10-20 uM calcium) but becomes extensively toxic when these traces of calcium were removed by 100 uM EGTA.

It has been suggested that active metabolites from drugs and chemicals may damage the plasma membrane, allowing calcium entry into the cell and resulting in cell death. These results clearly indicate, however, that the toxicity of the three compounds does not require the entrance of external calcium. If calcium mediates cell injury with these compounds then mobilization and redistribution of intracellular calcium must be involved.

Significance to Biomedical Research and Program of the Institute. Acetaminophen is a commonly used analgesic. Severe overdose is associated with hepatotoxicity and nephrotoxicity in humans, presumably resulting from the generation of active metabolite. However, little to nothing is known concerning the biochemical events involved in the development of injury. The finding that acetaminophen induced toxicity could be produced by calcium may help in the elucidation of acetaminophen and other drug-induced toxicities.

Proposed Course of Project: The changes in calcium movement in cells treated with acetaminophen will be studied using the dye Arsenato III.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00848-02 LCP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Evidence by SEM for the mediator role of calcium in hepatotoxicity

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

P.I.:	Mariam George	Vist. Fellow	LCP	NHLBI
	Richard Chenery	Vist. Fellow	LCP	NHLBI
	Gopal Krishna	Chief, Section	LCP	NHLBI
Other:	None			

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Drug-Tissue interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

We have shown previously that a combination of ionophore A23187 and calcium induced marked morphological changes in the structure of cultured hepatocytes as seen by scanning electron microscopy (SEM). We have extended our observations on these structural changes by utilizing transmission electron microscopy (TEM). The numerous balloons and blebs observed when cells were treated with calcium ionophore A23187 in the presence of calcium primarily involved the plasma membrane; blebs contained only a pale matrix largely devoid of cytoplasmic organelles. The major cell organelles appeared intact although damaged cells exhibit swollen mitochondria. Comparison with the toxicity induced by 2,4-dinitrophenol (DNP) indicated that although ionophore and calcium cause some uncoupling of oxidative phosphorylation and hence decreased ATP levels, this action is not sufficient to account for the observed toxicity.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: Calcium has been proposed as a mediator of drug-induced toxicity and we have previously reported that calcium potentiates CCl_4-induced damage in cultured hepatocytes. We have performed studies to relate the observed cell damage induced by the ionophore and calcium to changes in morphology seen by scanning electron monoscropy (SEM) and transmission electron microscopy (TEM) in an attempt to determine which events may be critical to cellular injury induced by calcium. Preliminary experiments suggested that uncoupling of oxidative phosphorylation could be responsible for injury induced by calcium and ionophore. A comparison was therefore made between injury induced by the ionophore, and 2,4-dinitrophenol (DNP) which is classical uncoupler of oxidation phosphorylation in liver mitochondria. We have also used ethionine, which is known to deplete ATP through a mechanism different from DNP in order to compare the ATP depletion with toxicity.

Methods Employed: Hepatocytes from adult rat liver were prepared as described in our last report and were plated on 25 cm Falcon bottles which had been pretreated with poly-L-lysine. Cells were cultured for either 1,10 or 24 hr in William's Medium E modified as described previously. Cells were then treated with 1 uM or 5 uM ionophore dissolved in either DMSO or ethanol. Incubation was carried out for 2 hr at 37°C; the incubation medium was either William's Medium E or an Hepes buffered saline containing 5 mM glucose. DNP and ethionine were dissolved directly in the incubation medium. Toxicity was assessed by determining lactic dehydrogenase (LDH) and glutamic-pyruvic transaminase (GPT) leakage with a Hewlett-Packard 8450A spectrophotometer. Calcium concentrations were determined using the calcium-sensitive dye Arsenaco III in the same instrument. The total fatty acid content of cells was measured before and after treatment with ionophore. Lipids were extracted into chloroform: methanol, reduced and cleaved by lithium aluminium hydride and then treated with heptofluorobutryic anhydride. The fatty alcohols were analyzed on a Hewlett Packard 5830 fitted with capillary columns and an electron capture detector. The fixation and preparation of samples for SEM and TEM was also described in our last year's report.

Major Findings: Ionophore A23187 and calcium induced enzyme leakage from cells which had been cultured for 1, 10 or 24 hr. The magnitude of the cell damage was apparently independent of the nature of the solvent used (DMSO or ethanol) but was greater in the buffered saline than in William's Medium E. This may be due perhaps to the presence of magnesium in the medium. Ionophore was not toxic at very low calcium concentrations (10-30 uM) but was toxic at physiological calcium concentrations (3.6 mM).

SEM of cells cultured 24 hr revealed flattened cells with normal calcium resulted in a rounding of the cell, loss of microvilli and the appearance of numerous blebs and balloons which were continuous with the plasma membrane. TEM showed that the blebs contained a clear matrix and were largely devoid of cytoplasmic organelles. These changes were surface membrane and some microvilli. Treatment with ionophore and

associated with significant enzyme leakage in the order of 30% but no injury to major cell organelles was apparent, except for some swelling of the mitochondria. Swollen mitochondria with a pale matrix were typically seen with uncouplers of mitochondrial oxidative phosphorylation.

Incubation of cells in the presence of DNP, a well-known uncoupler of oxidative phosphorylation, resulted in observable cytotoxicity at about 10^{-4}M. Moreover, at concentrations of DNP which caused no observable enzyme leakage, potentiation of cell damage induced by ionophore and calcium was observed. This potentiation of enzyme leakage was associated with increased morphological changes in mitochondrial structure and ballooning of the plasma membrane.

Ethionine decreased levels of ATP in liver cells by conversion to S-adenosyl ethionine thus reducing adenosine availability. Although no toxicity was observable with ethionine up to concentrations of 25 mM a distinct potentiation of DNP-induced injury was observed. This was consistent with injury resulting from ATP depletion. However, ethionine had no apparent effect upon injury induced by ionophore and calcium, indicating that the mechanisms by which ionophore and DNP induced cell injury were quite distinct. These observations were confirmed by the direct measurement of ATP in cells. ATP levels in control cultures were in the range of 2 mM. Treatment with 10^{-4}M DNP, however, reduced ATP levels to 1% of control (20 uM) within 20 min. After a similar period of incubation, ionophore in low calcium media caused no apparent change in ATP levels was detected. Incubation with 3.6 mM $CaCl_2$ and 1 uM ionophore resulted in a decrease in ATP levels to 40% of control, suggesting that uncoupling of oxidative phosphorylation occurred in calcium mediated injury but that the magnitude of the effect is insufficient to account for the observed cell injury.

Treatment of cells with 5 uM ionophore resulted in no change in total fatty acid content as judged by gas chromatographic analysis (within 1 hr of treatment). Changes in free fatty acid within the cell, however, may not be detected by this approach. The extracted lipids demonstrated no detectable change in diene conjugation after treatment with ionophore indicating no measureable lipid peroxidation was induced by ionophore.

Significance to Biomedical Research and Program of the Institute: Calcium has been suggested to mediate both drug-induced toxicity and ischemic injury. Studies with SEM and TEM provide a good qualitative impression of injury which relates well to quantitative parameters such as leakage of cytoplasmic enzymes. In addition significant information has been obtained concerning possible mechanisms by which calcium causes cell injury.

Propsoed Course of Project: We propose to examine the accumulation of calcium within specific organelles, e.g., endoplasmic reticulum and mitochrondia, during drug-induced toxicity, and to further investigate changes in plasma membrane during calcium induced injury.

Publications:

Chenery, R., George, M. and Krishna, G.: The effect of ionophore A23187 and calcium on carbn tetrachloride induced toxicity in cultured rat hepatocytes. Toxicology and Applied Pharmacology, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00849-01 LCP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Characterization of prostacyclin receptors in platelets

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

P.I.:	Gopal Krishna	Chief, Section	LCP	NHLBI
Other:	Nancy Kim	Chemist	LCP	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Drug-Tissue Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Purified plasma membranes prepared from human platelets were utilized to study the occurrence of specific prostacyclin (PGI_2) receptors in blood platelets of a number of individuals. The specific binding of [^{3}H] PGI_2 to platelet membranes were utilized to quantitate the number of PGI_2 receptors and their affinity to PGI_2. There were large variations in the number of PGI_2 receptors on 25 individuals so far tested. Attempts are presently being made to correlate these differences with the ability of PGI_2 to induce cyclic AMP formation and to inhibit platelet aggregation. Various components of anacardic acid were found to inhibit the specific binding of PGI_2 to the platelet membranes. The triene component was found to be more active than diene component and diene component was more active than monoene component.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: We reported earlier that prostacyclic [PGI_2] induced a marked increase in cyclic AMP in human blood platelets. Since there were large variations in the effect of PGI_2 among a number of individuals, we investigated if these were due to differences in the number of PGI_2 receptors on blood platelets. Since anacardic acid compounds inhibit PGI_2 induced cyclic AMP formation in platelets, we have tested their ability to displace PGI_2 from its receptors.

Method Employed: Purified plasma membranes were prepared from human blood platelets. The membranes were incubated with [^3H]-PGI_2 (2.5×10^{-8}M) for 1-5 min and membrane bound PGI_2 was separated by millipore filtration or by airfuge centrifugation. Nonspecific binding of [^3H]-PGI_2 was determined in each set of experiments by addition of 10^{-4}M PGI_2. Both millipore filtration and airfuge centrifugation gave identical results. The ability of PGI_2 to displace [^3H]-PGI_2 was employed as a measure of affinity of PGI_2 to the receptor.

Major Findings: The extent of [^3H]-PGI_2 binding to platelet membranes varied greatly among 25 individuals so far tested. These variations appeared to mainly be due to differences in the number of receptors rather than the affinity to PGI_2 (dissociation constant 10^{-7}M). Even though these studies are not complete, the differences of PGI_2 action as estimated by increases in cAMP or by inhibition of platelet aggregation in these individuals appear to correlate with the difference in the amount of [^3H]-PGI_2 bound to the receptor. Anacardic acid inhibited PGI_2 binding in a dose-dependent fashion. The triene component was found to be more active than the diene component and the diene component was more active than monoene component. These effects of the anacardic acid components correlated with their inhibitory effects on PGI_2 action in blood platelets.

Significance to Biomedical Research and Program of the Institute: The finding that individual variations in the PGI_2 may be due to differences in the number of PGI_2 receptors rather than the affinity of PGI_2 receptor suggests that individuals may be classified as low, medium or high responders to PGI_2 based on the ability of PGI_2 to inhibit platelet aggregation and to increase cyclic AMP. The development of a specific inhibitor such as anacardic acid may be useful in the study of this role of PGI_2 in platelet function.

Proposed Course of Project: We propose to continue to investigate the difference in the number of PGI_2 receptors and platelet function. We also propose to correlate the differences in PGI_2 receptors with the occurrence of specific HLA antigens on platlets as well as lymphocytes of various individuals. We also propose to investigate other specific but more potent inhibitors of PGI_2 receptors.

Publications:

Lloyd, H.A., Denny, C. and Krishna, G.: A simple liquid chromatography method for analysis and isolation of unsaturated compounds of anacardic acids. Journal of Liquid Chromatography 3 (10), 1497-1504, 1980.

Kaushal, D.C., Carter, R., Miller, L.H. and Krishna, G. Gametocytogenesis by malaria parasites in continuous culture, Nature 286: 490-492, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00850-01 LCP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Inhibition of DNA synthesis by nitroso chloramphenicol: Protection by GSH

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

P.I.:	Gopal Krishna	Chief, Section	LCP	NHLBI
	David Siegel	Guest Worker	LCP	NHLBI
Other:	None			

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Drug-Tissue Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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)

Earlier we have shown that nitroso chloramphenicol does not induce aplastic anemia in calves or mice. We have investigated the mechanism by which GSH prevents the inhibition of DNA synthesis by nitroso chloramphenicol in bone marrow cells. GSH reacts nonenzymatically with nitroso chloramphenicol within seconds to form a glutathione conjugate which is converted to the hydroxyamine analogue of chloramphenicol. Preincubtion of nitroso chloramphenol with GSH completely prevents the inhibition of DNA synthesis induced by the compound indicating the GSH conjugate and the hydroxylamine derivation are not responsible for the inhibition. The ineffectiveness of nitroso chloramphenicol to induce aplastic anemia in animals may be due to conversion of nitroso chloramphenicol to compounds which are not inhibitors of DNA synthesis.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: We reported last year that nitroso chloramphenicol did not cause aplastic anemia in mice or calves. One of the main objections of this study has been to investigate in vitro the mechanism by which nitroso chloramphenicol could lead to bone marrow damage and to determine if the damage can be reversed by GSH, which is present in red cells and other tissues. Preliminary studies indicated that GSH reverses inhibition of DNA synthesis in bone marrow cells induced by nitroso chloramphenicol. We have also investigated the mechanism by which GSH reacts with nitroso chloramphenicol to form compounds that do not inhibit DNA synthesis.

Methods Employed: DNA synthesis in bone marrow cells was measured by incorporation of ^{3}H thymidine into DNA. The cells were preincubated with ^{3}H-thymidine for 15 min prior to addition of nitroso chloramphenicol. The reaction of nitroso chloramphenicol with GSH was followed spectrophotometrically as well as chromatographically.

Major Findings: Nitroso chloramphenicol caused a dose-dependent inhibiton of DNA synthesis. Half maximal inhibition was attained at about 30 uM. The inhibition of DNA synthesis was readily prevented by pre-incubation of nitroso chloramphenicol with equi-molar amounts of GSH. The reaction of nitroso chloramphenicol with GSH at pH 7.4 occurred within seconds. The GSH conjugate then decomposes to a hydroxylamine analogue and chloramphenical. At these concentrations, neither GSH conjugate nor hydroxylamine analogue was effective as an inhibitor of DNA synthesis. Even though nitroso chloramphenicol is very effective in inhibiting DNA synthesis in the bone marrow cells, the inhibiton does not appear to be related to its conversion to by hydroxylamine analogue.

Significance to Biomedical Research and to Program of the Institute: The major finding that GSH reacts with nitroso chloramphenicol to yield derivatives that do not inhibit DNA synthesis in the bone marrow may explain the inability of nitroso chloramphenicol to cause bone marrow damage in vivo.

Propsoed Course of Project: We propose to study the inhibition of DNA synthesis in bone marrow cells in vitro with other nitro and nitroso compounds in order to determine the possible mechanism for the inhibition of DNA synthesis by nitroso chloramphenicol. This should aid our understanding of the mechanism of bone marrow damage induced by chloramphenicol and possibly other nitro compounds.

Publications:

Krishna, G., Aykac, I. and Siegel, D.: Recent studies on the mechanism of chloramphenicol activation responsible for aplastic anemia. In Safety Problems Related to Chloramphenicol and Thiamphenicol Therapy, Y. Najean, Tognoni, G. and Yunis, A., eds., Raven Press, 1981 - pp.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00856-02 LCP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Studies on the renal reabsorption of cephalosporins in the rat

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

P.I.:	Harriet M. Maling	Chief, Physiology Sect.	LCP	NHLBI
	James R. Gillette	Chief, Lab. of Chem. Pharm.	LCP	NHLBI
OTHER:	Wilford Saul	Chemist	LCP	NHLBI

COOPERATING UNITS (if any)

Mrs. Annie Arvidsson, Dept. of Clinical Pharmacology, Karolinska Inst., Huddinge, Sweden

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Physiology Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	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)
Urinary flow rates, urinary urate concentrations, and urinary urate excretion rates were compared in 3 groups of anesthetized (sodium pentobarbital) female Sprague-Dawley rats before and for 3 hr after i.v. injections of saline, cephaloridine (250 mg/kg) or probenecid (250 mg/kg). Cephaloridine approximately doubled the rate of urine flow during the first hour after injection. Although the urinary urate concentration did not change significantly, the urinary urate excretion rate increased from 0.77 ug/min to 1.4 ug/min (6 rats). This "uricosuric effect" of cephaloridine may be the result of the diuresis. The doubling of the urate excretion rate was much less than the 5 to 10-fold increases which occurred in rats injected with probenecid.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: The objective of this project is to determine the types of endogenous transport systems which may be involved in the reabsorption of cephaloridine in the rat. The first endogenous transport system to be examined was the urate reabsorption transport system.

Methods Employed: In female Sprague-Dawley rats anesthetized with sodium pentobarbital, the urinary bladder was catheterized with a poyethylene tubing inserted through the urethra. Urine was collected during successive one-hour periods. After a one-hour control period, each rat was injected in the tail vein with saline, cephaloridine or probenecid. Urine was then collected during three successive one-hour periods. In some experiments, blood samples were taken from a retro-orbital sinus. Urate concentrations were measured by a uricase method with a Sigma kit.

Major Findings. In a series of experiments in which each rat served as its own control, the urinary excretion rate and clearance of urate during 135 min of saline infusion before the i.v. injection of cephaloridine (40 mg/kg) were significantly lower than during the 135 min of saline infusion following the injection of cephaloridine. Although these findings suggested a uricosuric effect of cephaloridine, other interpretations were also possible, such as diurnal variations, effects of the anesthetic, effects of the saline infusions, and effects of frequent blood samples. Control experiments in which some of these factors were varied convinced us that cephaloridine should be injected i.v. in a group of rats and compared in the same experiment with a group of rats which received only a saline injection, and with another group of rats which was injected i.v. with probenecid, a universally recognized uricosuric drug. Cephaloridine (250 mg/kg i.v.) approximately doubled the rate of urate excretion during the first hr after injection. Although the urinary urate concentrations were not increased by this dose of cephaloridine, the urate excretion rate increased, probably because of the diuresis, from 0.77 to 1.5 ug/min. In contrast, probenecid (250 mg/kg i.v.) did not cause a diuresis, but increased the urinary urate concentration 4 to 8-fold and the excretion rate 5 to 10-fold.

^{14}C-Uric acid was not taken up by rat renal cortical slices. Uptake of uric acid did occur with rabbit cortical slices. Cephaloridine (10^{-4} and $3x10^{-4}M$) depressed uric acid uptake by 16 and 33%. Cephapirin ($3x\ 10^{-5}$, 10^{-4}, and $3x10^{-4}M$) depressed ^{14}C-uptake by 41 to 58%, almost as much as probenecid (60-64% at 3×10^{-5}, 10^{-4} and $3 \times 10^{-4}M$) The significance of the depression of uric acid uptake by rabbit renal slices is not clear. Since the tubular lumens are known to be collapsed in kidney slices, the significance of measurements of uptake of uric acid is dubious.

Significance to Biomedical Research and the Program of the Institute. Our initial findings suggest that the uric acid transport system might involved to a slight extent in the reabsorption of cephaloridine. However, the correlation with diuresis makes this possibility unlikely.

Proposed Course of Project: This project will be terminated.

Publications:

Maling, H.M., Saul, W.,Yasaka, W.J. and Gillette, J.R.: Effects of isoproterenol on the toxicity in rats of compounds eliminated by the kidneys. *Pharmacology* 21:256-267, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00857-02 LCP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Steady-state pharmacokinetics of p-aminohippurate (PAH) in rats

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

P.I.:	Harriet M. Maling	Chief, Physiology Sect.	LCP	NHLBI
	James R. Gillette	Chief, Lab. of Chem. Pharm.	LCP	NHLBI
Other:	Wilford F. Saul	Chemist	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Physiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	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)

A new principle for estimating hepatic blood flow rates is demonstrated in steady-state experiments in rats in which p-aminohippurate, labeled with either ^{14}C or ^{3}H, was infused into the portal vein, with simultaneous infusion into the tail vein of the same compound labeled with the other isotope. Hepatic blood flow and hepatic extraction ratios were calculated from measurements of urinary and biliary excretion rates and blood concentrations of each isotope. The principle may be used to calculate hepatic blood flow from measurements of urinary excretion and systemic blood concentrations of drug and metabolite after intravenous injections of radiolabeled drug and precursor.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: The objective of this study is to demonstrate with radiolabeled PAH the validity of some pharmacological relationships derived for steady-state conditions during the simultaneous infusion into the portal vein of a compound labeled with one isotope and into a systemic vein of the same compound labeled with a different isotope. Measurements of steady-state blood concentrations and urinary and biliary excretion were used in the calculation of hepatic blood flow.

Methods Employed: In female rats, radiolabeled (^{14}C or ^{3}H) PAH was infused at a constant rate for 120-140 min into the portal vein and PAH labeled with the other isotope was infused simultaneously at the same rate into the tail vein. ^{3}H and ^{14}C-radioactivity were measured in blood samples from the retro-orbital sinus and in urine and bile collected from polyethylene catheters inserted into the bladder through the urethra and into the bile duct.

Major Findings. Hepatic blood flow varied greatly in different rats, from a low value of 12.4 ml min^{-1} kg^{-1} to a high of 106.4 ml min^{-1} kg^{-1}; the mean hepatic blood flow in 5 rats was 56.9 ± 16.0 ml min^{-1} kg^{-1}. In the same 5 rats, the renal blood flow, as estimated from the PAH renal clearances, averaged 22.3 ± 1.77 ml min^{-1} kg^{-1}. Measurements in individual rats suggest that the renal blood flow is only about 1/3 to 1/2 the hepatic blood flow in rats. As expected, the hepatic extraction ratios of PAH were low, less than 6%. In individual rats, the extraction ratios ranged from 0.012 to 0.053.

Significance to Biomedical Research and Program of the Institute. This study represents an initial step in the development of relatively noninvasive procedures for estimating hepatic blood flow. Biliary excretion was used as a substitute for a substance that is rapidly excreted into urine and is also partially converted into a metabolite by the liver. An intraportal infusion of a test substance may be replaced by an intravenous infusion of a precursor that is eliminated solely by conversion to the test substance in liver. Exploration of these approaches leads to theoretical pharmacokinetic relationships among precursor, metabolite, and test substance. It becomes apparent that hepatic blood flow can be calculated from measurements in blood and urine of radioactivity in precursor, test substance and metabolite which is derived from intravenous administration of appropriately radiolabeled test substance and precursor.

Proposed Course of Project: A search will be made for appropriate precursor-drug-metabolite combinations which can be used in making estimates of functional hepatic blood flow.

Publications:

Gillette, J.R., Saul, W.F. and Maling, H.M.: A new principle for estimating hepatic blood flow rates. Pharmacology, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00917-06 LCP

PERIOD COVERED

October 1, 1980 to September 30, 1981

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

P.I.:	Jack A. Hinson	Sr. Staff Fellow	LCP	NHLBI
	Terrence J. Monks	Vist. Fellow	LCP	NHLBI
	Lance R. Pohl	Sr. Staff Fellow	LCP	NHLBI
Others:	None			

COOPERATING UNITS (if any)

Dr. Robert J. Highet is in the Laboratory of Chemistry, NHLBI. Catherine C. Fenselau is at the Middle Atlantic Mass Spectrometry Laboratory at Johns Hopkins University.

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Enzyme-Drug Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The hepatotoxicity of acetaminophen is believed to be mediated by the reactive metabolite, N-acetylimidoquinone. At therapeutic doses of acetaminophen, the reactive metabolite is efficiently detoxified by reaction with glutathione (GSH). An acetaminophen-GSH conjugate is believed to be formed but definitive proof of its structure has not yet been presented until now. Acetaminophen was administered to rats and a metabolite believed to be acetaminophen-GSH conjugate was isolated from bile by preparative high pressure chromatography. The ^{13}C NMR and field desorption mass spectometry of the metabolite confirmed that it was a GSH conjugate of acetaminophen. A ^{1}H NMR investigation of the metabolite established uniquivocally that the GSH-conjugate of acetaminophen is 3-(glutathion-S-yl) acetaminophen. This finding is consistent with N-acetylimidoquinone being the reactive metabolite of acetaminophen that reacts with liver GSH, and other tissue molecules.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: Previous studies in our Laboratory have shown that the hepatotoxicity of acetaminophen is mediated by a reactive metabolite that can react covalently with various tissue components including liver glutathione (GSH). The objective of this project is to determine the structure of the GSH conjugate of acetaminophen. This information should help define more clearly the structure and the possibe mechanism of formation of the reactive metabolite of acetaminophen.

Methods Employed: Acetaminophen was administered (500 mg/kg, i.p.) to rats and their bile was collected for up to 24 hr. The GSH conjugate of acetaminophen was isolated from the bile by preparative HPLC and its structure was determined by field desorption mass spectrometry, ^{13}C NMR and ^{1}H NMR.

Major Findings: The ^{13}C NMR and field desorption mass spectra (MH+ at 457 a.m.u.) of the isolated bile metabolite confirmed that it was a conjugate of GSH and acetaminophen. The structure of the metabolite was defined as 3-(glutathion-S-yl) acetaminophen by ^{1}H NMR spectrometry.

Significance to Biomedical Research and Program of the Institute: The identification of 3-(glutathion-S-yl) acetaminophen as a metabolite of acetaminophen in vivo is consistent with N-acetylimidoquinone being a reactive metabolite of acetaminophen. The results of previous work indicates that this metabolite is formed from acetaminophen by a direct oxidation. N-Acetylimidoquinone can react with a variety of tissue nucleophiles. It appears to react with GSH in its 3 position. This interaction probably explains, at least in part, how acetaminophen depletes liver GSH.

Proposed Course of Project: The project is terminated.

Publications:

Hinson, J.A., Pohl, L.R., Monks, T.J., Gillette, J.R. and Guengerich, F.P.: 3-Hydroxyacetaminophen: A microsomal metabolite of acetaminophen. Evidence against an epoxide as the reactive metabolite of acetaminophen. Drug Metab. Disp. 8: 289-294, 1980.

Hinson, J.A., Pohl, L.R., Monks, T.J. and Gillette, J.R.: Acetaminophen-induced hepatotoxicity. Life Sci.29: 107-116, 1981.

Gillette, J.R., Nelson, S.D., Mulder, G.J., Jollow, D.J., Mitchell, J.R., Pohl, L.R. and Hinson, J.A. Formation of chemically reactive metabolites of phenacetin and acetaminophen. In Proceedings of the Second International Symposium on Biological Reactive Metabolites. D. Parke, J.J. Kocsis, and R. Snyder (eds.), Plenum Press, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00927-01 LCP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Interactions of doxorubicin with canine pulmonary microsomes

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

P.I.:	Henry A. Sasame	Chemist	LCP	NHLBI
Others:	None			

COOPERATING UNITS (if any)

Dr. Michael R. Boyd, Clinical Pharmacology Branch, National Cancer Institute, Bethesda, Md. 20205

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Enzyme-Drug Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The rate of adrenochrome formation from epinephrine by dog lung microsomes and NADPH was increased 3-fold by doxorubicin and was completely blocked by superoxide dismutase. The rate of hydrogen peroxide formation by dog lung microsomes was increased 5-fold by the drug and was virtually obliterated by catalase or a nitrogen atmosphere.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: One major problem that occurs during the course of treatment of sarcoma (bone sarcoma) patients is that metastases of the sarcoma invade pulmonary tissue and cause death. In order to treat the pulmonary metastases, NCI has recently initiated a new approach in the administration of doxorubicin. Cne lung is unilaterally cannulated and perfused with a given concentration of doxorubicin, while the other lung is kept completely intact. Preliminary studies have shown that doxorubicin does not enter the systemic system. Although most of the toxicity of doxorubicin is due to myocardial necrosis, it is also possible that the drug might cause pulmonary toxicity. Since the primary cause of toxicity by doxorubicin is thought to be mediated through the formation of superoxide anion, our initial studies will be focused on the formation of superoxide by isolated dog lung microsomes.

Methods Employed: Dog lung tissues were cut into small pieces and homogenized by three 10 sec bursts, in a Waring blender followed by polytron in 0.02 M Tris pH 7.4-1.15% KCl containing 15% glycerol plus 1 mM dithiothreitol, 1 mM EDTA. Following centrifugation at 18,000 x g for 20 min, the supernatant fraction was centrifuged at 100,000 x g for 75 min. Lung microsomes were washed twice with Tris-KCl buffer. Adrenochrome formation was assayed in an Aminco DW II spectrophotometer.

Major Findings: 1) Although lung microsomes from dogs lack cytochrome P-450 the NADPH cytochrome c reductase activity is about 14 nmoles/mg/min. 2) Doxorubicin enhanced the rate of adrenochrome formation from epinephrine by NADPH with dog lung microsomes and the enhancement was virtually abolished by the presence of superoxide dismutase, indicating the presence of superoxide anion. 3) Doxorubicin also caused a 5-fold increase the rate of H_2O_2 formation by dog lung microsomes which was completely abolished by either the presence of catalase or a nitrogen atmosphere.

Significance of Biomedical Research to the Program of the Institute: Biochemical studies on dog lung microsomal interaction with doxorubicin are vitally important particularly, if the new perfusion technique will be used clinically to treat metastasized sarcoma patients. Comparative biochemical studies between cardiac and pulmonary microsomes may provide leads to the mechanism of toxicity elicited by doxorubicin.

Proposed Course of Project: Oxygen uptake and NADPH oxidation by dog lung microsomes and the level of superoxide dismutase and catalase in dog lung will be compared with those in heart soluble fraction. Moreover, antioxidants such as vitamin C, p-hydroxytoluene will be used to block superoxide anion formation.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00931-02 LCP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Stereospecific dealkylation of oxprenolol by mixed-function oxygenases

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

P.I.:	Henry A. Sasame	Chemist	LCP	NHLBI
Others:	None			

COOPERATING UNITS (if any)

Wayne Levine, Hoffmann-La Roche, Nutley, New Jersey and Wendel Nelson, University of Washington, Seattle, Washington.

LAB/BRANCH

Laboratory of Chemcial Pharmacology

SECTION

Enzyne-Drug Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS: 0.8	PROFESSIONAL: 0.8	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)

An antibody against cytochrome P-450_b (induced by phenobarbital) did not significantly inhibit the O-dealkylation, N-dealkylation, and ring hydroxylation of oxprenolol by liver microsomes from untreated rats, or rats treated with phenobarbital or B-naphthoflavone. An antibody against cytochrome P-450_c (induced by 3-methylcholanthrene) did not inhibit the reactions by liver microsomes from untreated rats or phenobarbital treated rats but inhibited all three reactions by liver microsomes from rats treated with B-naphthoflavone, which also induces cytochrome P-450_c synthesis.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: Last year we reported that the relative rates of oxidation of the S and the R isomers of oxprenolol depended not only on the animal species but also on the type of oxidation as well as the treatment of rats with phenobarbital (PB) or B-naphthoflavone (BNF). These observations lead me to believe that several forms of cytochrome P-450 are responsible for the difference in the relative rates. In order to evaluate this hypothesis, the effects of antibodies against different forms of cytochrome P-450 on the different reactions were studied with microsomes from rats treated with PB or BNF.

Methods Employed: Varying amounts (0.5, 5, 12 mg/nmole P-450) of antibody specific to either P-450_b (induced by PB) or P-450_c (induced by 3-MC) were preincubated at room temperature for 10 min and then incubated with oxprenolol as previously described in last year's report.

Major Findings: 1) An antibody against cytochrome P-450_b (induced by PB) did not significantly inhibit the formation of desisopropyl oxprenolol (DIOP), or desallyl oxprenolol (DAOP) by liver microsomes from either untreated rats or rats treated with PB or BNF. Similarly this antibody did not inhibit the formation of 4-hydroxy oxprenolol (4-HOP) by liver microsomes from untreated rats or rats treated with BNF. As reported last year, the formation of 4-HOP is not catalyzed by liver microsomes from rats treated with PB.

Since the treatment of rats with PB increases the rates of formation of DAOP and DIOP per mg of microsomal protein PB apparently induces an unidentified cytochrome P-450 that catalyzes the N-dealkylation and O-dealkylation of oxprenolol.

2) An antibody against cytochrome P-450_c (induced by 3-MC) did not inhibit the reactions by liver microsomes from control rats or PB treated rats but inhibited all three reactions by liver microsomes from BNF treated rats.

Maximum inhibition of the metabolism of the R form ranged from 45% for DIOP to about 75% for 4-HOP, whereas maximum inhibition of the metabolism of the S form ranged from 20% for DAOP and 55% for 4-HOP.

3) The treatment of rats with BNF doubled the rates of formation of both DIOP and 4-HOP per nmole of P-450 in microsomes but decreased the rate of formation of desallyloxprenolol to about one-half that of the control.

Significance to Biomedical Research and to the Program of the Institute: In view of the wide use of this B-adrenergic blocker as an antihypertensive drug, its metabolism by different forms of cytochrome P-450 by hepatic microsomes is of interest. Moreover, the use of stereoisomers should provide a better insight of the specificity of the cytochrome P-450 enzymes.

Proposed Course of Project: Since we have initiated a similar research project using propranolol, this project will be terminated.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00932-02 LCP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

New metabolites of chloramphenicol

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

P.I.:	Terrence R. Burke, Jr.	Guest Worker	LCP	NHLBI
	Patricia Morris	Biologist	LCP	NHLBI
	Lance R. Pohl	Sr. Staff Fellow	LCP	NHLBI

Others: None

COOPERATING UNITS (if any)

Dr. Terrence R. Burke, Jr. is a Research Associate in the Pharmacology-Toxicology Research Associate Program, NIGMS, Bethesda, Md. 20205

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Enzyme-Drug Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.20	1.20	

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)

Cytochrome P-450 of rat liver metabolized chloramphenicol (CAP) into p-nitrobenzyl alcohol ($NO_2C_6H_4CH_2OH$) and N-(2-hydroxyethyl)-dichloroacetamide ($HOCH_2CH_2NHCOCHCl_2$). These products appeared to be formed by the oxidative cleavage of a carbon-carbon bond of CAP. This reaction may represent a previously uncharacterized pathway of metabolism by cytochrome P-450. Under conditions of low oxygen tension, cytochrome P-450 of rat liver catalyzed the dechlorination of CAP to monodeschloro-chloramphenicol. Either monodeschloro-chloramphenicol or an intermediate produced during its formation appeared to be reactive enough to bind covalently to microsomal protein. Neither chloramphenicol amine nor any other nitro reduction product of CAP was produced by rat microsomes. In contrast, gut bacteria reduced CAP to the amine.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: To identify microsomal and gut flora metabolites of chloramphenicol CAP, characterize the enzymes that metabolize CAP to these metabolites, and determine whether a metabolite is responsible for the aplastic anemia produced by CAP.

Methods Employed: CAP labeled with ^{14}C in the dichloroacetamido carbons and ^{3}H in benzylic position was incubated with rat liver microsomes or intestinal contents. After 30 min, the reactions were stopped by the addition of methanol. The methanol supernatants were analyzed by HPLC and the metabolites were identified by mass spectrometry. Covalent binding to microsomal protein was determined by a standard method.

Major Findings: When CAP was incubated with gut material under anaerobic conditions only one major metabolite was detected by HPLC. This compound was identified as the p-aminophenyl derivative of CAP (CAP amine) by mass spectrometry.

Several metabolites of CAP were detected by HPLC when CAP was incubated in air with rat liver microsomes from phenobarbital (PB) treated rats in the presence of a NADPH generating system. Two of these metabolites, p-nitrobenzyl alcohol and CAP oxamic acid, have been previously identified. A third metabolite has now been identified as N-(2-hydroxyethyl)-dichloroacetamide ($HOCH_2CH_2NHCOCHCl_2$) by mass spectrometry. Since the formation of the metabolites was inhibited by more than 70% when the incubations were conducted without NADPH, in the presence of SKF 525-A, or under an atmosphere of N_2 or $CO:O_2$ (8:2), cytochrome P-450 catalyzes the oxidative formation of all of the metabolites. Moreover, the metabolites could not be detected when the incubations were performed with microsomes from untreated rats or rats treated with B-naphthoflavone.

The incubation of CAP with microsomes from PB pretreated rats under an atmosphere of nitrogen resulted in the formation of only one metabolite that was identified as the monodechlorinated metabolite of CAP (monodeschloro-CAP) by mass spectrometry. Cytochrome P-450 appeared to catalyze the formation of this metabolite since very little of the compound was detected by HPLC when the incubations were conducted in the presence of SKF 525-A or under an atmosphere of 100% CO. The metabolite was not detected when the incubations were conducted with microsomes from untreated rats or rats treated with B-naphthoflavone. The formation of monodeschloro-CAP was not affected by the presence of 0.5 or 1 mM GSH in the reaction mixture.

^{14}C and ^{3}H CAP were also found to bind covalently to microsomal protein anaerobically in a ratio of 1:1. Cytochrome P-450 appeared to catalyze the reaction; both SKF 525-A, and 100% CO blocked the reaction. GSH did not affect the covalent binding. In addition, virtually no binding to protein was detected when the incubations were conducted with microsomes from untreated rats, or rats treated with B-naphthoflavone.

Significance to Biomedical Research and Program of the Institute: In this investigation we found that rat liver cytochrome P-450 metabolized CAP into p-nitrobenzyl alcohol ($NO_2C_6H_4CH_2OH$) and $HOCH_2CH_2NHCOCHCl_2$. These products appear to have been formed by an oxidation reaction which resulted in the cleavage of a carbon-carbon bond of CAP. There is no precedence for such a reaction catalyzed by cytochrome P-450. Therefore, by studying the mechanism of this reaction, we can learn more about a fundamental catalytic activity of cytochrome P-450.

Cytochrome P-450 also appears to cleave reductively one chlorine from CAP to produce monochloro-CAP. This reaction occurs most rapidly under low oxygen tension. Since the formation of monochloro-CAP correlates with the covalent binding of ^{14}C and ^{1}H-CAP under anaerobic conditions it might be possible that monochloro-CAP or an intermediate in the formation of monochloro-CAP is responsible for the covalent binding of CAP to microsomal protein. In either case, the monodechlorination of CAP appears to produce a reactive and possibly toxic metabolite of CAP. In this regard, CAP is known to inhibit irreversibly the metabolism of other drugs; it might be possible that during the monodechlorination of CAP by cytochrome P-450 a reactive metabolite is formed which binds covalently to cytochrome P-450 and consequently inactivates it.

Other investigators have previously suggested that the nitro group of CAP is reduced to an amine by liver microsomes. But, our results indicate that liver microsomes do not reduce CAP to any of the nitro reduced products including nitroso-CAP (NO-CAP), CAP-hydroxylamine (NHOH-CAP) or CAP amine (NH_2-CAP). We have found unequivocal evidence, however, that gut bacteria reduce CAP to its amine.

Since other aromatic amines are known to be toxic, particularly after they have been further oxidized by microsomal enzymes, CAP amine should be considred a potential toxic metabolite of CAP. This idea is supported by the fact that thiamphenicol, the p-methylsulfonylphenyl derivative of CAP ($CH_3SO_2C_6H_4R$), cannot be metabolized to an aromatic amine and has never been reported to produce aplastic anemia.

Proposed Course of Project: We plan to 1) characterize the mechanism by which cytochrome P-450 cleaves a carbon-carbon bond of CAP, 2) to characterize the mechanism of the monodechlorination activation of CAP by cytochrome P-450 and 3) to investigate the metabolism of CAP amine in order to determine whether is metabolized into a bone marrow toxin.

Publications:

Martin, J.L., Gross, B.J., Morris, P. and Pohl, L.R.: Mechanism of glutathione-dependent dechlorination of chloramphenicol and thiamphenicol by cytosol of rat liver. Drug Metab. Disp. 8: 311-375, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00935-02 LCP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Induction of enflurane defluorination by hydrazine containing compounds

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

P.I.:	Richard Branchflower	Staff Fellow	LCP	NHLBI
	Lance R. Pohl	Sr. Staff Fellow	LCP	NHLBI
Other:	John W. George	Chemist	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Enzyme-Drug Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.29	.0 4	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)

Pretreatment of rats (and other rodents) with isoniazid or hydrazine results in an increased rate of defluorination of the general anesthetic enflurane by liver microsomes. Analysis of liver microsomes by SDS gel electrophoresis or DE-52 anion exchange chromatography did not reveal any differences in either the amount or type of cytochrome P-45) caused by treatment with either compound. Analysis of microsomal lipids indicates that there are no apparent differences between lipids extracted from the microsomes of control rats and those from pretreated animals. However, analysis by DE-53 anion exchange chromatography suggests the presence of a "new" minor band of cytochrome P-450 in the microsomes obtained from the livers of rats treated with hydrazine. These results indicate that a new form of cytochrome P-450 might be responsible for the increased rate of metabolism of enflurane to the potential kidney toxin F^-. Whether or not other hydrazine containing drugs such as hydralazine, carbidopa, procarbazine, and phenylbutazone induce a similar form of cytochrome P-450 remains to be determined.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: A recently reported case of F^- induced kidney toxicity following enflurane administration is thought to have been associated with previous chronic administration of isoniazid. Indeed, we and other workers have demonstrated that pretreatment of rats with isoniazid leads to increased *in vitro* defluorination of enflurane to F^-. The objective of the current investigation is to characterize further the biochemical nature of this potentiating effect, to determine whether it is a generalized property of drugs and environmental pollutants containing the hydrazine moiety and to what extent do hydrazines affect the metabolism and/or toxicity of other drugs.

Methods Employed: Liver cytochrome P-450 catalytic activity was assayed by measuring the metabolism of enflurane to F^-. Cytochrome P-450 and cytochrome P-450 reductase were partially purified by anion exchange chromatography. The reductase was further purified by affinity chromatography. Neutral and polar lipids were analyzed by tlc. Phospholipids and di- and triglycerides were hydrolyzed to their constituent fatty acids which were esterified and analyzed by GC. Partially purified cytochrome P-450 was recombined with cytochrome P-450 reductase and dilaurylphosphatidyl choline and incubated with enflurane.

Major Findings: Extraction of liver microsomes from control and hydrazine treated rats with butanol/acetone (to remove lipid) resulted in total loss of defluorinase activity which could be reconstituted to only control levels by addition of synthetic dilaurylphosphatidylcholine. Yet no differences were detected between the neutral or polar lipids extracted from the rat liver microsomes of control and hydrazine treated animals. Neither was there any detectable difference in the fatty acid composition of the microsomal lipids as determined by GC. DE-53 column anion exchange chromatography of detergent solubilized microsomes from control and hydrazine treated animals revealed the presence of a distinctive though minor "new" form of cytochrome P-450 in the microsomes from hydrazine treated animals. When the detergent was removed in preparation of the recombination with reductase and lipid this "new" form was readily converted to cytochrome P-420. Reconstitution experiments with this enzyme resulted in enflurane defluorinase activities only slightly higher than those obtained from control enzymes. Thus it would appear that hydrazine induces a minor form of cytochrome P-450 which is unusually susceptible to degradation when it is removed from its lipid environment. Induction of enflurane defluorination by hydrazine was demonstrated in the following species: Sprague-Dawley rats, Fischer rats, Golden Syrian hamsters, multicolor guinea pigs, Swiss-Webster NIH mice, C-57Bl/610 mice and DBA/2 mice. Although no increase in defluorinase activity was detected when New Zealand white rabbits were treated with a single dose of hydrazine more extensive investigations are needed to determine whether or not this species will make a good model for studying the mechanism of the induction process.

Significance to Biomedical Research and Program of the Institute: These results indicate that the increased defluorination of enflurane produced by isoniazid might be produced by its metabolite hydrazine. At the present time this effect appears to be due to the induction of a "new" minor form of cytochrome P-450. Other hydrazine containing drugs such as hydralazine, carbidopa, procarbazine, and phenylbutazone might increase the metabolism of enflurane to F^- by a similar mechanism.

Proposed Course of Project: The immediate direction of the research will focus on attempts to reconstitute the "new" form of cytochrome P-450 without converting it to its inactive form and thus to determine whether this enzyme is responsible for the increase in enflurane defluorination. Further work will be directed in the following areas. 1) Characterization of the mechanism of induction by cytochrome P-450. (Possibly by development of the New Zealand rabbit as a genetic model of noninducibility.) 2) The characterization of cytochrome P-450 inducing effects of hydrazine containing drugs e.g., hydralazine, carbidopa, procarbazine and phenylbutazone.

Publication:

Burke, T.R., Branchflower, R.V., Lees, D.E. and Pohl, L.R.: Mechanism of defluorination of enflurane: Identification of an organic metabolite in rat and man. Drug Metab. Disp. 9: 19-24, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00938-01 LCP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Mechanisms of formation of glutathione conjugates of bromobenzene

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

P.I.:	Terrence J. Monks	Vist. Fellow	LCP	NHLBI
Others:	Jack A. Hinson	Sr. Staff Fellow	LCP	NHLBI
	Lance R. Pohl	Sr. Staff Fellow	LCP	NHLBI
	James R. Gillette	Chief	LCP	NHLBI

COOPERATING UNITS (if any)

Dr. Robert J. Highet, Laboratory of Chemistry, NHLBI

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Enzyme-Drug Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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)

Five hr after administration of bromobenzene (500 mg/kg i.p.) to rats, hepatic GSH levels are depleted by 75%. Two bromobenzene-GSH conjugates have subsequently been found in bile. Isolation and purification of these conjugates by HPLC and analysis by ^{13}C and ^{1}H nuclear magnetic resonance spectroscopy revealed these metabolites to be trans-3-bromo-6 (glutathion-S-yl)-cyclohexa-2,4-dien-1-ol) and trans-4-bromo-6(glutathion-S-yl)-cyclohexa-2,4-dien-1-ol. The same two conjugates were formed in rat liver microsomes in the presence but not in the absence of 100,000 g supernatant of liver. Results with phenobarbital and 3-methylcholanthrene pretreated rats indicate that both conjugates are formed via the same reactive intermediate, namely bromobenzene 3,4-oxide.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: Bromobenzene is converted to a 3,4-oxide and to either a 2,3- or 1,2-oxide, as evidenced by the formation of both ortho and para-bromophenol. Pretreatment of rats with 3-methylcholanthrene increases the formation of ortho-bromophenol whereas pretreatment of rats with phenobarbital increases the formation of para-bromophenol. However, formation of the ortho-bromophenol apparently is via a nontoxic pathway whereas induction of the para-bromophenol pathway increases toxicity. The objective of the present study was to determine the relative activities of the two bromobenzene oxides and their relationship to GSH depletion, which preceeds the hepatic necrosis caused by bromobenzene.

Methods Employed: The two bromobenzene-GSH conjugates were isolated from the bile of rats receiving bromobenzene (500 mg/kg, i.p.), purified by HPLC and analyzed by ^{13}C and ^{1}H nuclear magnetic resonance spectroscopy. The two conjugates were also identified as *in vitro* metabolites of bromobenzene in standard microsomal incubations containing 100,000 x g rat liver supernatant. Rats were pretreated with either phenobarbital or 3-methylcholanthrene to observe the effects on both the *in vivo* and *in vitro* formation of the two conjugates.

Major Findings: Bromobenzene was found to be metabolized to two GSH conjugates identified as trans-3-bromo-6 (glutathion-S-yl)-cyclohexa-2,4-dien-1-ol) and trans-4-bromo-6 (glutathion-S-yl)-cyclohexa-2,4-dien-1-ol which are subsequently excreted into bile in a ratio of 1.6:1, respectively. This ratio is constant over a 10-fold dose range (50-500 mg/kg) and unaffected by pretreatment of rats with either phenobarbital or 3-methylcholanthrene. However, treatment with phenobarbital caused an increase in the total amount of conjugate excreted into bile whereas treatment with 3-methylcholanthrene caused a slight decrease. Similarly, in rat liver microsomes from phenobarbital treated rats containing 100,000 x g supernatant, there was a 6-fold increase in the rate of formation of the two conjugates (2.44 ± 0.20 nm/mg/min and 1.87 ± 0.16 versus 0.37 ± 0.01 and 0.33 ± 0.1 for controls) but a 25-50% decrease in conjugate formation with microsomes from 3-methylcholanthrene treated animals (0.28 ± 0.01 and 0.17 ± 0.02 nm/mg/min). In the absence of 100,000 x g supernatant from control rats no conjugate formation could be observed.

Significance to Biomedical Research and Program of the Institute: The finding that bromobenzene 3,4-oxide gives rise to two bromobenzene-GSH conjugates yet bromobenzene 2,3 or 1,2-oxide does not, explains how the 3,4-oxide pathway is toxic and yet the 2,3 or 1,2-oxide pathway is not. Thus, formation of GSH conjugates eventually lead to depletion of hepatic GSH and subsequent liver necrosis. That the 2,3- or 1,2-oxide apparently does not form a GSH conjugate indicates that this pathway will not lead to GSH depletion, perhaps because they rapidly decompose to the phenol.

Proposed Course of Project: We intend to extend this study to isolate and identify other GSH conjugates of chemically reactive intermediates and to determine which of the many GSH-S-transferase enzymes are responsible for catalyzing the detoxification of these intermediates.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 H1 00939-01 LCP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Stability of reactive intermediates of bromobenzene

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

P.I.:	Terrence J. Monks	Vist. Fellow	LCP	NHLBI
	Serrine S. Lau	Vist. Fellow	LCP	NHLBI
Other:	James R. Gillette	Chief	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Enzyme-Drug Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.25	0.25	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Bromobenzene toxicity is manifested by both liver and kidney necrosis and also by the covalent binding of radiolabeled bromobenzene to lung tissue. We have shown that a reactive metabolite of bromobenzene, namely the 3,4-oxide generated by hepatocytes, is sufficiently stable to leave cells by trapping it with either externally added protein or with externally added glutathione and glutathionyl transferases. Thus, this intermediate may be responsible for the extrahepatic toxicity observed following bromobenzene administration.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: After the administration of radiolabeled bromobenzene, covalently bound label is found not only in the liver but also in a number of other tissues and blood plasma. The finding of covalently bound label in plasma raises the possibility that reactive intermediates of bromobenzene metabolism are sufficiently stable to leave the liver and be carried by blood to other organs.

The present study was therefore undertaken to determine whether or not bromobenzene reactive metabolites are capable of leaving isolated rat liver hepatocytes *in vitro*.

Methods Employed: Isolated rat liver hepatocytes were prepared by a two-stage collagenase perfusion technique as previously described by this laboratory. Bromobenzene metabolites were assayed by HPLC. To this end an assay for the phenolic metabolites of bromobenzene was devised. The method depends on extraction into ether followed by back-extraction into 0.5 N NaOH. The extract is neutralized and assayed by HPLC at 254 nm with water, methanol, acetic acid (53.5:45.0:1.5 v:v) as the mobile phase. Conjugated metabolites of bromobenzene were assayed by extraction into ice-cold acetone followed by centrifugation and evaporation of the supernatant to dryness under nitrogen. The residue was dissolved in water and assayed by HPLC.

Hepatocytes were prepared from control, phenobarbital treated and 3-methylcholanthrene treated rats. The 100,000 x g supernatant was prepared from control rats only.

Major Findings: Isolated rat liver hepatocytes metabolize bromobenzene (1 mM) to two GSH conjugates and to both ortho and para-bromophenol. Dimethyl maleate treatment (620 mg/kg) of phenobarbital induced rats decreased bromobenzene GSH conjugate formation by 90 to 95%, but caused only a 31% increase in para-bromophenol values. Addition of 1 mM GSH and 100,000 x g supernatant to these hepatocyte suspensions caused a 13 to 25-fold increase in conjugate formation. Moreover, in hepatocytes from rats pretreated with phenobarbital and diethyl maleate, the addition of GSH caused a 29% decrease in para-bromophenol formation, addition of 100,000 x g supernatant (2 ng protein/ml) a 46% decrease (albumin at equivalent concentration caused a 39% decrease) and addition of GSH and 100,000 x g supernatant combined caused a 90% decrease. Similar additions to hepatocyte suspensions from 3-methylcholanthrene pretreated rats had no significant effect on ortho-bromophenol formation. The results suggest that bromobenzene 3,4-oxide is sufficintly stable to leave the cell, whereas bromobenzene 1,2 or 2,3-oxide rearranges to the phenol prior to leaving the cell.

Signifiance to Biomedical Research and to the Program of the Institue: The finding that bromobenzene 3,4-oxide is sufficiently stable to leave the cell offers an explanation for the extra-hepatic necrosis observed following bromobenzene administration. It also explains why pretreatment of rats with phenobarbital potentiates this toxicity whereas pretreatment with 3-methylcholanthrene offers a degree of protection.

Proposed Course of Project: We intend to define better the relationship between the formation of bromobenzene-GSH conjugates, formation of the bromophenols and covalent binding in isolated rat hepatocytes. We shall also investigate whether reactive intermediates of other compounds leave cells in which they are generated.

Publictions: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00940-01 LCP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Electrophilic halogens as potential toxic metabolites of halocarbons

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

P.I.:	Lance R. Pohl	Sr. Staff Fellow	LCP	NHLBI
	Richard V. Branchflower	Staff Fellow	LCP	NHLBI
Other:	John W. George	Chemist	LCP	NHLBI

COOPERATING UNITS (if any)

Dr. Robert J. Highet is in the Laboratory of Chemistry, NHLBI.

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Enzyme-Drug Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	0.6	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)

When carbon tetrachloride (CCl_4), bromotrichloromethane ($CBrCl_3$), chloroform ($CHCl_3$) or carbon tetrabromide (CBr_4) were incubated with liver microsomes from phenobarbital pretreated rats in the presence of 2,6-dimethylphenol (DMP), analysis of the reaction mixture by gas chromatography mass spectrometry revealed that 4-chloro-2,6-dimethylphenol (ClDMP) was a metabolite of CCl_4 and $CBrCl_3$, whereas 4-bromo-2,6-dimethylphenol (BrDMP) was a metabolite of CBr_4. The formation of the metabolites decreased to undetectable levels when the reactions were conducted with heat-denatured microsomes, in the absence of NADPH, or in an atmosphere of N_2. These results indicate that the chlorines of CCl_4 and $CBrCl_3$ and the bromines of CBr_4 are oxidatively metabolized by rat liver microsomes to reactive and potentially toxic electrophilic metabolites such as Cl_2, HOCl, Br_2, or HOBr, that are trapped by DMP to form ClDMP or BrDMP.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: In order to determine whether the hepatoxic compounds CCl_4, $CBrCl_3$, or CBr_4 are metabolized to electrophilic chlorine or broomine species, they have been incubated with rat liver microsomes in the presence of 2,6-dimethylphenol (DMP) which is known to readily react with halogenating agents.

Methods Employed: 4-Chloro-2,6-dimethylphenol (ClDMP) was synthesized by mixing DMP with sulfonyl chloride and its structure determined by ^1H NMR. Liver microsomes were prepared from Sprague Dawley rats that had been pretreated with phenobarbital. CCl_4, $CHCl_3$, $CBrCl_3$, and CBr_4 (5 mM) were incubated for 30 min with liver microsomes (2 mg/ml), a NADPH generating system, and DMP (5 mM). The formation of ClDMP and 4-bromo-2,6-dimethylphenol (BrDMP) were determined by gas chromatography mass spectrometry.

Major Findings: When CCl_4, $CBrCl_3$ or CBr_4 were incubated with rat liver microsomes for 30 min in the presence of DMP, analysis of the reaction mixture by gas chromatography mass spectrometry revealed that ClDMP was a metabolite of CCl_4 and $CBrCl_3$, whereas BrDMP was a metabolite of CBr_4. In contrast, $CHCl_3$ was not metabolized to ClDMP. The order of the amounts of metabolites produced from CCl_4, $CBrCl_3$, and CBr_4 were $CBr_4 > CBrCl_3 > CCl_4$. The formation of the metabolites decreased to undetectable levels when the reactions were conducted with heat denatured microsomes, in the absence of NADPH, or under atmosphere of N_2.

Significance to Biomedical Research Program of the Institute: We have previously shown that CCl_4 and $CBrCl_3$ are metabolized *in vitro* in presence of air and *in vivc* to phosgene ($COCl_2$). One possible way for $COCl_2$ to be produced from CCl_4 and $CBrCl_3$ is for these compounds to be initially reduced to $CHCl_3$, followed by oxidation of the C-H bond of $CHCl_3$ to $COCl_2$. However, since CCl_4 does not appear to be metabolized to $CHCl_3$ in the presence of air, $COCl_2$ must be formed by another mechanism during in the aerobic metabolism of CCl_4 or $CBrCl_3$.

In the present investigation we have discovered another oxidative metabolite of CCl_4 and $CBrCl_3$. The isolation and identification of ClDMP indicates that the new metabolite is an electrophilic chlorine which is trapped by reaction with DMP. In the case of CBr_4, an electrophile bromine appears to be formed.

A potential mechanism that might explain these observations involves the initial reductive dehalogenation of tetrahalomethane (CX_4) to trihalomethyl radical ($CX_3\bullet$). Trichloromethyl radical ($CCl_3\bullet$) is known from chemical studies to react with molecular oxygen at nearly diffusion control rates to form trichloromethylperoxy radical ($CCl_3OO\bullet$) which appears to decompose to produce $COCl_2$ and an unidentified electrophilic form of chlorine which has been proposed to be hypochlorous acid (HOCl) and/or molecular chlorine (Cl_2). If a similar series of reactions occurred in microsomes, this would explain, at least in part, how CCl_4 and $CBrCl_3$

are metabolized to $COCl_2$ and an electrophilic chlorine. An analogous set of reactions with CBr_4 would lead to $COBr_2$ and HOBr, or Br_2.

The previously unreported pathway of metabolism of CCl_4, $CBrCl_3$, and CBr_4 described in this report might be responsible, at least in part, for the toxicity produced by these compounds in-as-much as HOCl, HOBr, Cl_2, and Br_2 are known to be toxic oxidizing agents.

Proposed Course of Project: We plan to determine the mechanism of oxidation of halocarbon compounds to electrophilic halogen metabolites. The studies will include: 1) determining what enzyme(s) in liver microsomes catalyzes the reaction, 2) characterizing the mechanism of action of the enzyme(s), 3) determining the substrate specificity of the enzyme(s) and 4) studying the potential involvement of this metabolic pathway in the toxicity produced by halocarbon drugs and environmental chemicals.

Publications:

Pohl, L.R., Branchflower, R.V., Highet, R.J., Martin, J.L., Nunn, D.S., Monks, T.J., George, J.W., and Hinson, J.A.: The formation of diglutathionyl dithiocarbonate as a metabolite of chloroform, bromotrichloromethane, and carbon tetrachloride. Drug Metab. Disp. in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00941-01 LCP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Effects of ketones on cytochrome P-450

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

P.I.:	Richard V. Branchflower	Staff Fellow	LCP	NHLBI
	Lance R. Pohl	Sr.Staff Fellow	LCP	NHLBI
Other:	John W. George	Chemist	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Enzyme-Drug Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.31	.06	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)

Several aliphatic ketones to which humans are exposed are known to potentiate the toxicities produced by various xenobiotics including chloroform and carbon tetrachloride. We investigated the possibility that the potentiation may be due to increases in the amount of specific forms of cytochrome P-450 that metabolize the toxicants. In support of this view treatment of animals with methyl butyl ketone (MBK) increased the total amount of cytochrome P-450 in the liver and induced a form of cytochrome P-450 that was similar in molecular weight to that induced by phenobarbital (PB). DE-52 anion exchange chromatography further indicated the similarity in the forms of cytochorme P-450 induced by MBK and PB. Moreover, microsomes from rats treated with MBK or PB metabolized $CHCl_3$ to $COCl_2$ more rapidly than did microsomes from untreated rats. Pretreatment of rats with MBK also potentiated the hepatotoxicity and renal toxicity produced by $CHCl_3$. Thus MBK apparently potentiates the toxicity of $CHCl_3$ by increasing the amounts of a form(s) of cytochrome P-450 that metabolizes $CHCl_3$ to the toxic metabolite $COCl_2$. Other ketones, such as acetone, may also change the composition of liver cytochrome P-450.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: The goal of this project is to determine whether ketones potentiate the toxicity of xenobiotics by inducing the synthesis of specific forms of cytochrome P-450 that catalyze their metabolism to toxic metabolites.

Methods Employed: Microsomes, cytochrome P-450, DE-52 anion exchange column chromatography, the assay of the glutathione adduct of phosgene, SDS polyacrylamide electrophoresis, and SGPT and BUN determinations were performed by published methods.

Major Findings: Pretreatment of rats with MBK for 3 days resulted in an increase of liver cytochrome P-450 from 0.6 to 0.9 nmol/mg protein. DE-52 anion exchange chromatograms of liver microsomes from rats pretreated with MBK were very similar to the chromatograms of microsomes from phenobarbital (PB) pretreated rats. SDS polyacrylamide electrophoresis revealed that microsomes from MBK and PB pretreated rats contained a major protein fraction of molecular weight of approximately 48,000. Microsomes from rats pretreated with MBK or PB metabolized $CHCl_3$ to $COCl_2$ at approximately 5-fold higher rate than did microsomes from untreated rats. Moreover, rats pretreated with MBK or PB were significantly more susceptible to the hepatotoxicity produced by $CHCl_3$ than were untreated rats. Although pretreatment of rats with MBK also potentiately the renal toxicity of $CHCl_3$, it did not appear to affect the level of renal cytochrome P-450. In addition, $CDCl_3$ was less renal toxic in MBK pretreated rats than was $CHCl_3$.

Significance to Biomedical Research and the Program of the Institute: The results of the present study indicate that MBK potentiates the hepatotoxicity produced by $CHCl_3$, at least in apart, by inducing a form(s) of cytochrome P-450 in liver microsomes that metabolizes $CHCl_3$ to $COCl_2$ significantly more rapidly than does cytochrome P-450 from microsomes of untreated rats. Although it is not clear how MBK potentiates the renal toxicity of $CHCl_3$, the finding that $CDCl_3$ was less renal toxic than was $CHCl_3$ suggests that a metabolite is also involved in this toxicity. Other ketones, such as acetone, to which humans are exposed may also induce changes in the composition of liver cytochrome P-450 and thereby affect the rate of metabolism and toxicity of a variety of drugs.

Proposed Course of Project: The immediate course of this investigation will be: 1) to reconstitute the protein bands from the DE-52 anion exchange column in an attempt to determine which one is responsible for the metabolism of $CHCl_3$ to phosgene, 2) to determine whether MBK induces the same forms of cytochrome P-450 as does PB, 3) to extend these studies to acetone and other ketones and 4) to determine the mechanism by which aliphatic ketones induce cytochrome P-450, 5) to determine the mechanism by which MBK potentiates the renal toxicity of $CHCl_3$.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00942-01 LCP

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Development of a flow cell to detect cytochrome P-450

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

P.I.:	Richard V. Branchflower	Staff Fellow	LCP	NHLBI
	Lance R. Pohl	Sr. Staff Fellow	LCP	NHLBI

Others: None

COOPERATING UNITS (if any)

Robert F. Bonner is in the Biomedical Engineering and Instrumentation Branch.

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Enzyme-Drug Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	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)

During the past 10 years there has been considerable interest in isolating and characterizing the numerous species of cytochrome P-450. Separations are most often accomplished by a combination of several types of column chromatography. Progress in this field, however, has been greatly hampered by the methodology for assaying <u>cytochrome P-450</u>. For example, the following steps are involved in an assay of a column fraction for cytochrome P-450. 1) The sample is reduced with sodium dithionite; 2) the reduced sample is added to a sample and reference cuvette, and a background spectrum is run; 3) carbon monoxide is bubbled through the sample cuvette, and a difference spectrum is recorded; 4) finally, the concentration of cytochrome P-450 is determined. We have designed a <u>flow cell</u> which in combination with a <u>microprocessor unit</u> may be used to assay cytochrome P-450 as it is eluted from columns. This innovation should increase the resolution and sensitivity of the various column chromatography procedures used in the characterization and purification of cytochrome P-450 and decrease the time required for analyses.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: To develop a flow cell microprocessor unit for the continuous monitoring of cytochrome P-450 levels in eluents from either low or high pressure liquid chromatography columns.

Methods Employed: The design of the initial detector includes a mixing chamber, flow cells, and microprocesser computer components.

Major Findings: The project has progressed through the design stage and a prototype is currently under construction.

Significance to Biomedical Research and Program of the Institute: Cytochrome P-450 has been demonstrated to play an important role in drug interactions, xenobiotic toxicity, carcinogenesis and regulation and synthesis of a number of endogenous compounds. It is suspected that different people have different compositions of cytochrome P-450. Thus, the development of an analytical technique that will facilitate the purification and characterization of the various forms of cytochrome P-450 will greatly aid us in determining the role of cytochrome P-450 in physiology and pathology.

Proposed Course of Project: The immediate course of this investigation involves the development of a prototype detector which would be used to determine optimal design parameters for both the flow cell, mixing chamber and the microprocessor unit. Once these parameters are determined a unit will be developed which will be compatible with a number of spectrophotometric detectors which are commercially available and are of varying degrees of sophistication.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00943-01 LCP

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Cultured rat hepatocytes in the study of drug-induced hepatotoxicity

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

P.I.:	Richard Chenery	Vist. Fellow	LCP	NHLBI
	Mariam George	Vist. Fellow	LCP	NHLBI
	Gopal Krishna	Chief, Section	LCP	NHLBI
Other:	None			

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Drug-Tissue Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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 rapid loss of cytochrome P-450 mediated drug metabolizing activities has greatly impended the use of cultured rat hepatocytes in studies of drug induced hepatotoxicoty. Recent improvements in culture conditions have enabled cytochrome P-450 to be maintained for 24 hr. We have confirmed these studies and have investigated the metabolism and toxicity of acetaminophen after various periods of culture. Three major metabolites of acetaminophen have been detected in the culture medium; glucuronide, sulphate and glutathione conjugates. Changes in the relative production of these three conjugates in cultures occurred after 1 day suggesting adaptation to the culture conditions. Experiments were performed with hepatocyes prepared from animals induced with phenobarbital in vivo. These induced cells maintained high levels of cytochrome P-450 after 24 hr and metabolized acetaminophen at rates which were similar but not identical to those found with fresh cells; and exhibited toxicity within 6 hr. Thus, hepatocytes cultured for 24 hr appear to be useful for studying acetaminophen and other drug-induced toxicities.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: Acetaminophen is a commonly used analgesic that causes liver necrosis in large doses. Cultured hepatotocytes have been suggested as a tool to investigate drug-induced toxicity. The toxicity of acetaminophen arises from metabolic activation to a reactive intermediate, which reacts with and depletes cellular glutathione. When sufficient glutathione is depleted the reactive intermediate increases and causes cell damage. The main objective of this study has been to establish culture conditions which would retain the cytochrome P-450 as well as conjugation pathways in cultured hepatocytes and to induce cell damage with acetaminophen in these cultures.

Methods Employed: Hepatocytes were isolated and plated as described in the last report with minor modifications. All cultured dishes were precoated with collagen. Two media were employed in this study. Qne was William's Medium E modified by replacing bicarbonate with 10 mM HEPES pH 7.4. This medium also contained 25 mM nicotinamide and 10% v/v horse serum. The second medium was as above but cysteine, and cystine were omitted and 100 uM hydrocortisone 21-sodium succinate and 100 uM amino-levulinic acid added whilst nicotinamide and 10% v/v horse serum were only present for the initial hour of culture.

Cytochrome P-450 was measured spectrally after solubilization in an Emulgen buffer. Metabolites of acetaminophen in the culture medium were measured by reverse phase HPLC and U.V. detection.

Major Findings: Primary hepatocytes cultured in either of the culture media maintained levels of cytochrome P-450 after 1 day. After this time levels of the cytochrome gradually decreased with time in the cultured hepatocytes. At 48 hr cytochrome P-450 levels were in the range of 55-65% of fresh cells.

The rates of appearance of metabolites of acetaminophen in the culture medium were linear for up to 6 hr with both cultured and fresh cells. The accumulation of metabolites was therefore measured over a 2 or 4 hr period. Cells cultured for 24 hr in medium containing nicotinamide produced glucuronide and glutathione conjugates at similar rates to fresh cells, whilst the appearance of sulfate was significantly reduced (71% of initial value). By 48 hr the production of sulfate was decreased to 56% of initial value whilst the formation of the glucuronide had increased to 129% of initial amounts. The rate of appearance of glutathione conjugate was similar to control values, despite the decrease in cytochrome P-450 levels.

Omission of cysteine, and cystine from the medium improved the general appearance of the cells. The formation appearance of the glucuronide decreased at 24 hr (63%) and then increased dramatically by 48 hr (189%). The metabolism to sulfate was less variable being retained at 89% after 48 hr. The appearance of glutathione conjugate showed a drop at 24 hr (64%) and then no further change at 48 hr. Hence in this case the metabolism through cytochrome P-450 is decreased relative to sulfation and glucuronidation at 48 hr.

Thus, in each case levels of cytochrome P-450 wereot significantly different from initial values but differences did occur in the metabolic profile of acetaminophen. Culture with nicotinamide maintained the rate of formation of the glutathione conjugate indicating that production of active metabolite was virtually unchanged during the initial 48 hr of culture.

Similarly hepatocytes have been cultured from animals treated with phenobarbital. Culture in medium containing 25 mM nicotinamide allowed cytochrome P-450 levels to be maintained for 24 hr at levels well above those found in hepatocytes from control animals. Moreover, the production of glucuronide was also maintained at or above levels present in freshly isolated cells even after 48 hr cultures. There was some decrease in sulfation after culturing cells for 24 or 48 hr, but the rate of formation of the glutathione conjugate was maintained in these cells.

Thus adult rat hepatocytes may be cultured for 24 hr and still retain the enzyme systems involved in the activation and detoxification of acetaminophen. Hepatocytes induced _in vivo_ with phenobarbital maintained elevated cytochrome P-450 and generate larger amounts of active metabolite. These functions were retained in 24 hr cultures. Such cells exhibited signs of cell injury within a short time when treated with high concentrations of acetaminophen (8 mM or 25 mM). The toxicity obtained _in vitro_ appears to correlate with _in vivo_ toxicity regarding the acetaminophen required as well as the requirement for the induction of cytochrome P-450.

Signifiance to Biomedical Research and Program of the Institute: Overdoses of acetaminophen have been associated with hepatotoxicity and nephrotoxicity in humans. This study shows that cultured hepatocytes are a good tool for the study of the metabolism and subsequent toxicity associated with this analgesic.

Proposed Course of Project: Culture conditions must be altered to prolong the time-span of hepatocytes with drug-metabolizing capability. The development of culture conditions compatible with _in vitro_ induction with phenobarbital would greatly facilitate studies on the mechanism of cytochrome P-450 induction, enabling the regulation and control of cytochrome P-450 isozyme composition to be studied in a defined system.

Publications: None

ANNUAL REPORT OF THE
LABORATORY OF CHEMISTRY
SECTIONS ON CHEMICAL STRUCTURE AND STRUCTURAL
NUCLEAR MAGNETIC RESONANCE
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

October 1, 1980, through September 30, 1981

The efforts of the Laboratory of Chemistry continue to be divided among two main activities, one chemical (Sections on Chemical Structure and Nuclear Magnetic Resonance) and the other biochemical (Section on Physiological Chemistry). The latter will be reviewed separately and appended to this report by its Section Head, Dr. J. Pisano.

In chemistry, our laboratory is currently active in three main areas: (1) isolation and analysis of biologically important compounds using gas and high pressure liquid chromatography, (2) structural analysis using mass spectrometry, nuclear magnetic resonance, X-ray single crystal methods, and (3) computer organization and analysis of scientific data.

Liquid chromatography is still an important activity in the lab; it is being used both analytically (analysis of verapamil and nifedipine, the latter by electrochemical detection) and preparatively (at short wavelength) in rat liver extracts, where it was used to prove the intermediacy of Δ^1-pyrroline in the metabolism of putrescine.

In mass spectrometry, the ^{252}Cf plasma desorption spectrometer built for us by Texas A&M is fully operational and valuable data has been obtained on digitonin, a dye-steroid complex, and a series of encephalin-related peptides. The necessary computer has been ordered, but until it arrives, the instrument will remain in Texas where samples will be run for us by the staff there.

Our GC-MS systems have finally been made to work with capillary columns by adding a new cooled inlet system that allows on-column injection. It is clear that both sensitivity and resolution will be much improved with this development.

The resurrected MS-9 mass spectrometer now operates in a much improved fashion and we are constructing a fast atom bombardment source that will allow polar molecules to be analyzed without heating the sample. Computerization of the instrument has not yet been successful due to the vendor's inability to supply appropriate programs.

In nmr spectrometry, the Nicolet high field supercon has afforded many excellent spectra this year, and it is being used widely by other groups, even for whole animal phosphorous studies (R. Balaban, NHLBI). Down time is about 20% and previous difficulties with the broad-bonded probe system have been largely resolved. In fact, all of our nmr spectrometers appear to be used increasingly for unorthodox studies: dye-nucleoside interactions, phosphate in bacteria, lipoprotein association, etc. Two-dimensional J-resolved nmr experiments

are being carried out routinely with this system, and it is clear that the increased information on spin couplings is very useful in structure determination.

One disappointment is that our older H-100 spectrometer run by a Digilab computer is no longer viable because the computer hardware is no longer supported by the manufacturer. Replacement of the computer plus interface will cost $50,000 and this is of doubtful economy considering the aged electronics and the high cost of maintenance likely. Replacement with a modern system, which could be very useful in relieving the pressure on the supercon, would be about $100,000.

The valuable assistance of Dr. James Ferretti (DCRT) is again gratefully acknowledged. He is a vital cog in the development and use of our nmr instruments.

In X-ray crystallography, Dr. J. Silverton has produced no less than seven complete structures this year, including a mycotoxin, a cardiac glycoside, a stable rotamer of a reticulene derivative, a synthetic acetylcholine inhibitor candidate, two unusual fused five-membered ring systems, and a small peptide. In one case, an R-factor of 5.2% was achieved on the wrong model. Such an R-factor is considered by many investigators to be incontrovertible, but its failure in this case justifies our laboratory's insistence on pursuing determinations to the limit of the data.

The NIH/EPA Chemical Information System has expanded somewhat since last year but our activities in its behalf are now largely managerial (Dr. G. W. Milne) and even these shortly will be taken over by the Office of the Director (Dr. C. Fisk). This very successful, though admittedly expensive, project initiated in our laboratory has therefore been terminated.

Alone or in collaboration (see individual reports for collaborators), members of the laboratory this year have: 1) discovered an interaction between quinacrine and adenosine phosphate using ^{31}P nmr, 2) studied the degree of association of lipoproteins in water via ^{1}H nmr, 3) examined with ^{31}P nmr growing _corynebacterium diphtheriae_, where the resonance lines were identified as orthophosphate (unless all diamines and calcium and magnesium are removed in which case pyrophosphate appears), 4) separated (HPLC) the components of _Anacardium occidentale_, one component of which is an inhibitor of prostacyclin receptors in platelets and a potent inhibitor of _B. glabrota_, a schistosomiasis-harboring snail, 5) discovered weaver ant (_P. simplex_) pheromones 4-heptanone, 6-methyl-5-hepten-2-one and -2-ol (GC/MS) possibly used in seasonal migrations, 5) discovered the structure of the anal gland secretion (a sex pheremone ?) (a 2-methyl-substituted long chain fatty acid) of the Israeli mongoose, _H. ichneumon_, important in rodent control in that country, 6) identified and quantitated, using deep UV detection HPLC, 2-pyrrolidone in rat liver and showed that it is derived from Δ'-pyrroline, 7) determined the crystal structure of racemic dimethyl tartrate and studied other crystalline forms to attempt an explanation of their peculiar melting point curves, 8) attempted crystallization of the liver mycotoxin, phomopsin, and determined (X-ray) the absolute stereochemistry of the tetracycline-related mycotoxin viridicatum

toxin to the 0.9999 level (CH 1-squared test), 9) elucidated the structures (X-ray) of two systems with 4 fused five membered rings; whose chemical reactivity is not in accord with the ring strain found and semi-theoretical analysis is continuing, 10) determined the structure (X-ray) of one of two stable rotational isomers of cis-6-bromo-N-formyl-norreticuline enabling inferences to be made concerning relative stability, 11) solved the structure (X-ray) of 7-butyl-8-hydroxy-1-azaspiro[5.5]undecane hydrobromide, related structurally to the acetylcholine inhibitor dihydroisohistrionotoxin, 12) solved the structure (X-ray) of the cardiac glycoside, humistratin containing a double bond impossible to locate with other techniques; 13) elucidated the structure of TBOC-$(gly)_3$-benzyl ester (X-ray) incorrectly assumed to be a nucleotide-peptide complex, 14) developed computer programs to handle weak reflections and to expedite data input for cell dimensions and structure solution, 15) elucidated the structures of two bile metabolites of bromobenzene and one of acetaminophen using J-resolved two dimensional Fourier transform nmr, 16) using ^{13}C nmr, established the structure of the chromophoric moiety of the anti-tumor drugs, chrysomycins A, B, IV and V, 17) developed an analytical scheme, using HPLC with electrochemical oxidation, for the cardiac drug, nifedipine, under study at NIH, 18) completed the synthesis of gastrolactone, the defensive constituent of _G. cyanea_, 19) identified (GC-MS) three unsaturated ketones, pheromones in _L. paessleri_, 20) characterized a series of synthetic peptides related to encephalin using ^{252}Cf plasma desorption mass spectrometry (CfPDMS) and elucidated the mechanism of peptide fragmentation with this new technique, 21) studied the structures (GC-MS) of sugars derived from HCG, 22) elucidated (MS, nmr, microreactions) the structure of a novel triterpene, 23) determined (CFPDMS) the nature of the highly soluble digitonin recovered from dimethylsulfoxide and characterized a steroid-dye complex used to label receptor sites, 24) identified glutamate as an impurity in hydroxyproline derived from proline, suggesting the presence of an additional metabolic pathway, 25) developed an analytical scheme for dicarboxylic acids in urine, resulting in identification of a carnitine-deficiency genetic disease, 26) analyzed products from a protein electrolysis system in an attempt to discover an intermediate responsible for mobilizing protons from water.

ANNUAL REPORT OF THE
LABORATORY OF CHEMISTRY
SECTION ON PHYSIOLOGICAL CHEMISTRY
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1980 to September 30, 1981

The Section studies normal and pathologic states in man at the molecular level. Two major projects are currently under investigation: (1) Chemistry and Biology of the Kallikrein-Kinin System; and (2) Discovery and Characterization of Naturally Occurring Biologically Active Peptides.

Kallikrein-Kinin System

Bradykinin is one of the most potent vasodepressor and pain-producing substances known. In blood, it is formed from the protein, kininogen, by the action of the enzyme, kallikrein. Kallikrein circulates as a zymogen, prekallikrein. We previously suggested that the hypotensive action of Plasma Protein Fraction (PPF), a clinically important plasma volume expander commonly used in surgery, was due to the presence of the prekallikrein activator, Hageman factor fragment (HF_f). Upon bolus IV injection of implicated lots of PPF, the patient's prekallikrein is activated and massive amounts of kinin are rapidly formed causing a precipitous fall in blood pressure. It is believed that some lots of PPF have produced lethal falls in blood pressure. We have obtained more convincing evidence that HF_f is the harmful agent in PPF, by using a newly discovered specific inhibitor of HF_f we isolated from corn. This inhibitor completely neutralized the hypotensive action of implicated lots of PPF and of purified HF_f.

Since there is a need for better animal models to study the pathophysiological significance of the kallikrein-kinin system, we were attracted to a recent report that the Brown Norway Rat is deficient in prekallikrein, kininogen, and Hageman factor. Unfortunately, our studies do not support this report, as we have found normal levels of the three proteins in the Brown Norway Rat.

In addition to lowering blood pressure and causing pain, bradykinin is thought to profoundly affect ion and water transport in the kidney. We previously reported that in addition to kallikrein, the kidney also contains kininogen and that both proteins are localized in cells of the distal nephron. Studies with kidney slices have shown that kallikrein concentration is highest in the outer cortex and decreases progressively from outer to inner cortex. We also discovered the zymogen, prokallikrein, in kidney homogenates and urine. Urinary prokallikrein has been further purified in order to study its mode of activation as there may be a rate-limiting and previously unreported, prokallikrein-activating enzyme under hormonal control.

Another area under investigation is renal lymph. It arises largely from the cortex (where kallikrein is concentrated) and flows at a uniquely high rate which is comparable to urine flow. To test the hypothesis that the kidney delivers kallikrein as well as renin to the blood, we have looked for kallikrein in lymph and found that it contains immunoreactive, but biologically inactive kallikrein. In addition to high levels of renin, renal lymph contains low levels of angiotensin converting enzyme (kininase II). Kallikrein and trypsin inhibitors are also present.

In our parallel study of naturally occurring inhibitors of kallikrein and Hageman factor, a new potent inhibitor of human polymorphonuclear (PMN) leucocyte elastase has been found in calla flower bulb extracts. The partially purified inhibitor is a much better inhibitor of this elastase than previously reported naturally occurring inhibitors and may be a useful new tool for studying the pathophysiological significance of PMN elastase.

Peptide Biochemistry

Convinced that numerous peptides with profound biological activities await discovery in man, we have embarked on a program to isolate and characterize peptides from naturally occurring rich sources such as insect and animal venom and amphibian skin. The rich sources are logical choices because it is highly probable that the structure of the peptides will be closely related if not identical with the peptides normally present in minute quantities in human tissues. The rationale includes: (1) Development of suitable micro-procedures for the isolation and characterization of peptides, (2) Development of biological screening tests (a critical step for the discovery of new peptides), (3) Production of antibodies to the new peptides, and (4) Use of the antibodies for the detection of cross-reacting peptides in man.

High performance liquid chromatography (HPLC) is a powerful new technique for isolation and characterization of peptides. We pioneered the application of the technique in the rapid identification and quantitation of picomol quantities of amino acid phenylthiohydantoins. Now we have refined the technique to make it more easily reproduced in other laboratories. The latest procedure involves step-wise rather than gradient elution and includes specific recommendation for the resolution of potentially troublesome derivatives. All common phenylthiohydantoins can be identified in a single 12-min run on a 15-cm column or, with slightly better resolution, in 14 min on a 25-cm column.

Many HPLC methods have been recommended for the analysis of peptides but none have been critically evaluated. We have compared numerous columns from several manufacturers using a variety of mobile phases and peptides. Best results (resolution, peak shape, sensitivity and recovery) were obtained with a mobile phase containing trifluoroacetic acid, triethylamine, water and acetonitrile and a C-18 column manufactured by Toyo Soda.

Antibodies to ranatensin (a peptide we previously isolated from frog skin), and to bombesin are forming in rabbits. We plan to use the antibodies to ranatensin to look for similar peptides in man.

Pilot studies on extracts of Gila monster (Heloderma suspectum) venom and venom sacs of the European hornet (Vespa crabro) have been encouraging. Gila monster venom contains a peptide that stimulates enzyme secretion from guinea pig pancreatic acinar cells probably by interacting with vasoactive intestinal peptide receptors. European hornet venom contains a peptide which causes depolarization of human neutrophil membranes without causing cell death. Furthermore, it causes the cells to secrete their granular contents suggesting a pre-inflammatory activity. Of no less interest, the peptide also blocks the activity of chemoattractants on these cells.

The amino acid composition of four peptides isolated from yellow jacket venom sacs has been determined. Three of the peptides lyse liposomes indicating that they are membrane-active and will cause mast cell degranulation.

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

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01002-08 CH

PERIOD COVERED

October 1, 1980 to September 30, 1981

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)

J. Osborne, MD IR NHLBI
L. Zech, MD IR NHLBI
J. Costa, M CN N

LAB/BRANCH

Laboratory of Chemistry

SECTION

Structural Nuclear Magnetic Resonance Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Phosphorus-31 nuclear magnetic resonance has been used to characterize the interaction between quinacrine and nucleotides. Phosphorus nmr also gives insight into the environment of the polyphosphate in corynebacterium diphtheriae. Proton magnetic resonance spectra of frozen VLDL samples may act as probes for the association within the lipoprotein.

PHS-6040
(Rev. 2-81)

Projects worked on during this year were: 1) binding studies of nucleotides with quinacrine and several cation species 2) proton magnetic resonance study of frozen lipoprotein samples 3) phosphorus magnetic resonance study of bacteria. 4) attempts to resurrect the XL-100-15 NMR-FTS spectrometer. 5) studies of chromaffin granules by ^{31}P nmr.

One of the greatest advantages of nuclear magnetic resonance is the ability to observe dynamic phenomena in solution. The interaction of chemical compounds with biologically important molecules like nucleotides is ideally suited to this research technique. We have been examining the interaction by quinacrine hydrochloride and adenosine phosphates by phosphorus-31 nmr. We have observed that the and phosphorus resonance of ATP give upfield chemical shift changes that are essentially linear with increasing concentrations of the quinacrine hydrochloride. When the ratio of 2:1 quinacrine to nucleotide is exceeded, the slope of the shift change diminishes, but does not reach a plateau. In the case of the terminal phosphates of both nucleotides, the initial aliquot of quinacrine causes the resonances of shift in the opposite direction and then to parallel the trend for the other resonances. It is difficult at present to interpret the results with any certainty because of the multiple equilibria that are involved. The simplest explanation would be that a 2:1 complex is being formed which does not directly involve the phosphate backbone. We are currently planning a computer model to simulate the data which should give us additional insight into the effects of the multiple equilibria on the resonance shifts.

We have always attempted to extend the nmr techique to characterize varied classes of biomolecules. Lipoproteins because of their large molecular weights and complexity pose a particularly interesting and difficult challenge. In the past, we have obtained chemical shift and relaxation data on both the lipid and protein portions of these compounds. We are now examining methods for studying the interactions between the various species of lipoproteins. One such method is the proton resonance spectra of frozen samples. The samples of triglyceride rich lipoprotein ULDL and chylomicrons were quick-frozen in liquid nitrogen and equilibrated at -35°C in the nmr probe. The integrated intensity of the observed signal can then be plotted as a function of the lipoprotein concentration in a series of dilution experiments. The rationale for this type of experiment lies in the fact that nmr is capable of obtaining signals only from mobil species. Water which is frozen in a crystal lattice would, therefore, give no signal while water which is "bound" to the lipoprotein would be excluded from the lattice and mobil within regions of the frozen material. It might also be expected that the percentage of the "bound" water would vary with the degree of molecular association of the lipoproteins. Two such experiments have thus far been performed, and indeed, the results suggest that the integrated signal (area) is not linear with concentration of the lipoprotein. The area of the residual signal was found to be greater than would be predicted on the basis of concentration of lipoprotein present, when expressed as triglyceride or cholesterol. Control experiments using deuterium oxide (D_2O) in place of water suggest that 60% of the residual signal was from "bound" water, the remaining 40% arising from fluid portions of the lipoprotein molecules.

Interests in applying nmr spectroscopy to *in vivo* systems is widespread in the research community. We share this interest, and are looking at one of the simplest systems, namely bacteria. *Corynebacterium diphtheriae* contain a region of high polyphosphate concentration which, if observed, should give valuable information applicable in biochemical research. We have examined samples of whole bacteria, sonicated samples, and isolated "inclusions" by phosphorus-31 nmr. Spectra in the presence of diamines and the amino acid arginine, in different phases of growth, with metal chelating agents (to minimumize the availability of the counter-cations calcium and magnesium) and with addition of the ionophore lasolocid A (X537A) (to remove the ions after the growth of the bacteria has been halted) have all been obtained. We are now in the process of characterizing the various resonance lines observed in the spectra. The results can be summarized as follows: 1) Most of the spectra have resonances consistent with orthophosphates, It is not unexpected but comforting that our instrumentation has sufficient sensitiviy and resolution for further *in vivo* studies 2) the polyphosphate resonance at +22 ppm is not observed in many of the spectra even though other techniques suggest they are present. This phenomenon is consistent with published reports and is important because one of our goals for these experiments was to determine conditions by which these signals can be obtained consistently. 3) The polyphosphate resonance is observed when the bacteria have a sufficient supply of diamines present and when the calcium and magnesium ions are removed from the sample. We speculate that these conditions allow the polyphosphates more mobility or fluidity within the confines of the "inclusion". Still remaining to be determined is whether some other processes like binding to paramagnetic ions or chemical exchange might cause the signal to be too broad for us to see in some of the spectra.

Attempts to resurrect the XL-100 nmr Fourier transform system have reached a conclusion. We had hoped that the additional facilities that this would provide would allow more concentrated studies on a number of projects. The results of the effort are the spectrometer portion of the system is operational but the Fourier transform portion, namely, the computer and associated electronics have aged past being of any further use.

PUBLICATONS: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01003-09 CH

PERIOD COVERED
October 1, 1980 - September 30, 1981

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)

LAB/BRANCH
Laboratory of Chemistry

SECTION
Chemical Structure Section

INSTITUTE AND LOCATION
NHLBI-NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

CHECK APPROPRIATE BOX(ES)

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

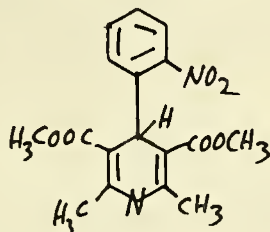
☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A scheme for the HPLC analysis of nifedipine has been developed. Gastrolactone from G. cyanea has been synthesized. A series of methylhex- and-heptenones has been identified in L. paessleri. A series of encephalin-related peptides, digitonin, and a drug-dye complex have all been successfully analyzed using ^{252}Cf plasma desorption mass spectrometry. The structure of a triterpene with a diene function has been elucidated. The structure of the major contaminant in HPLC columns has been elucidated. Glutamate has been identified in hydroxyproline from the peptide bound-proline reaction. Patients with carnitine deficiency have been found to produce large excess of succinate via ω-oxidation.

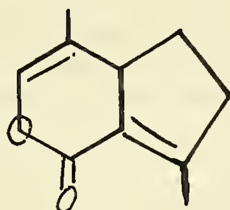
PHS-6040
(Rev. 2-81)

1) A scheme for the analysis in plasma of the calcium antagonist, nifedipine (1) has been developed (E. Speir Cardiology Branch, NHLBI) using high pressure liquid chromtography with electrochemical detection. A second more sensitive method using a nitrogen sensitive detector and gas chromatograph is under development.



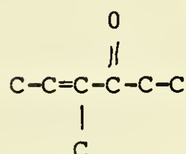
(1)

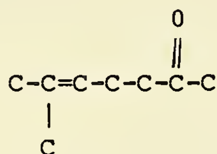
2) With T. Jones (Dept. of Entomology, Univ. of Ga), the synthesis of gastrolactone (2) a defensive constituent of Gastrophysa cyanea has been completed.

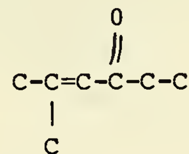


(2)

With G. Holmburg (Athabasca Univ.) a series of unsatured ketones (3, 4 and 5) have been identified as pheromones in L. paessleri.

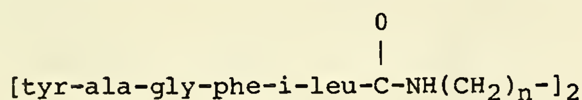






(3) (4) (5)

3) With Y. Shimohighashi and H. Chen (NICHD) a series of encephalin-related synthetic peptides (6) have been examined using ^{252}Cf plasma desorption mass

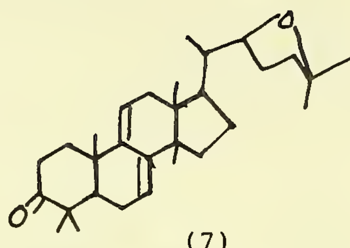


(6)

spectrometry. At masses up to m/z 1400, intact molecular ions are intense and cleavage occurs at each bond allowing the unequivocal assignment of peptide

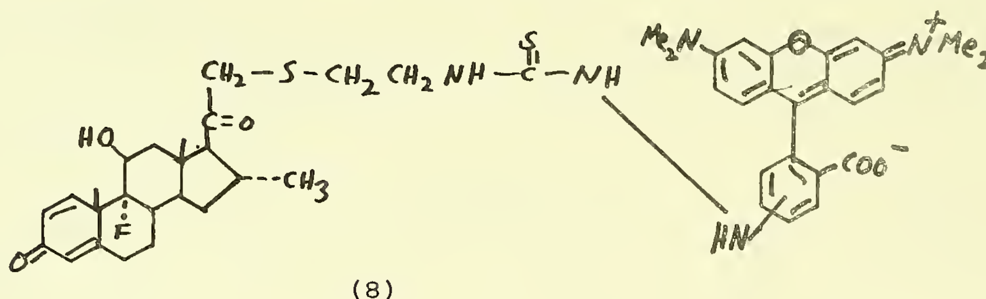
sequence. With this same group (Y.S., H.C.) a series of anisylidene sugars derived from the carbohydrate moeity of HCG have been investigated using GC-MS of silylated dervatives. Useful spectra have been obtained and model compounds are being studied to determine their structure.

4) With T. Waddell (U. of Tenn), the structure of less than 1 mg of a novel triterpene (7) has been elucidated using mass spectrometry, nmr, and microscale reactions (hydrogenation, methoxime formation, etc).



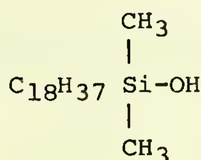
(7)

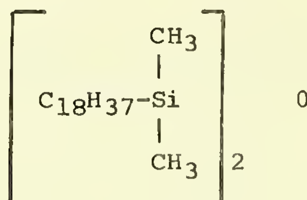
5) With H. Yeh (NIADDK) samples of digitonin recovered from dimethyl sulfoxide showing unusually increased solubility have been examined by ^{252}Cf plasma desorption mass spectrometry. Their spectra are identical with ordinary digitonin and it is concluded that their exceptional solubility is due to removal of traces of natural sterols (perhaps cholesterol) normally present by the solvent. Using the same technique, an excellent spectrum, confirming the structure of a steroid-dye complex used to label receptor sites, was obtained on (8). Impurities were also disclosed by the spectrum and the compound is presently being further purified (S. Simmons, NIADDK).



(8)

6) Using mass spectrometry and nmr, the structures of the major contaminents in HPLC columns was elucidated as octadecyldimethylsilanol and its ether (10,11) (P. Terry USDA, Beltsville).





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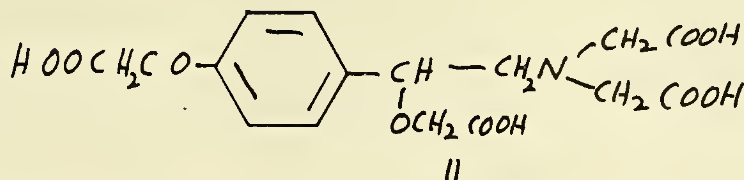
(9) (10)

7) Mass spectrometry and derivitization with silyl groups has been used to identify the important site-specific chelating agents synthesized by O. Gansen (NIADDK) i.e. (11), currently under study by NCI.



8) With B. Peterkofsky (NCI) an impurity complicating the determination of hydroxyproline (an indicator of peptide-bound proline) has been identified as glutamate by mass spectrometry and a series of simple reactions. Its source (from proline) is not clear from current metabolic theories and it may indicate a new pathway.

9) With B. Blumenkopf and K. Engel (NINCDS) an extensive series of GC/MS analyses have been carried out for dicarboxylic acids in urine, using a group of patients with carnitine deficiency. The disease can apparently be identified even in family members who are asymptomatic by noting sharp increases in succinate and other dicarboxylic acids on a simple diet scheme. The defect is clearly genetic.

10) With R. Hendler (NHLBI) an extensive series of GC-MS studies have failed to reveal any unusual consumption of reagent or appearance of a new product sought as the source of protons in mitochondrial transport. This negative result caused reexamination of an interpretation based on Mitchell's hypothesis of proton transport across cells.

The current proposal is that protons may be mobilized from water, even at potentials well below their redox potential, due to lack of oxygen in the system and other factors.

PUBLICATIONS

1. Jaouni, T. M., Leon, M. B., Rosing, D. R., and Fales, H. F. Analysis of verapamil in plasma by liquid chromatography. J. Chromatog. 182: 473-477, 1980.

2. Fales, H. M., Comstock, W., and Jones, T. H. Test for dehydrogenation in gas chromatography-mass spectrometry. Anal. Chem. 52: 980-82, 1980.

3. Hunninghake, G. W., Gadek, J. E., Fales, H. M. and Crystal, R. G. Human alveolar macrophage-derived chemotactic factor for neutrophils. J. Clin. Invest. 66: 473-483, 1980.

4. Fales, H. M., Jaouni, T. M., Schmidt, J. O., and Blum, M. S. Mandibular gland allomones of Dasymutilla occidentalis and other mutillid wasps. J. Chem. Ecol. 6: 895-903, 1980.

5. Jones, T. H., Blum, M. S., Fales, H. M., and Thompson, C. R. (5Z, 83)-3-heptyl-methylpyrrolizidine from a thief ant. J. Org. Chem. 45: 478-480, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01004-10 CH

PERIOD COVERED
October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)

Characterization of Natural Products

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

PI: H. A. Lloyd	Research Chemist	CH NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemistry

SECTION
Chemical Structure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The purpose of this work is to **determine** the **structures** of **physiologically active compounds** isolated from animal or plant tissue. High pressure liquid chromatography is used to purify the new compounds for nmr and mass spectrometry study.

PHS-6040
(Rev. 2-81)

Work on projects initiated last year was continued:

1. Components of cashew-nutshell extract Anacardium occidentale for pharmacological studies (in collaboration with Dr. G. Krishna, CP, NHLBI). Large scale isolation and purification of the substituted salcylic acids by high pressure liquid chromatography were investigated in order to have enough material to pursue the studies started last year. One of the components has interesting properties 6-(pentadec-8,11,143-trienyl) salicylic acid acts as a specific inhibitor of prostacyclin receptor in human blood platelets; it is also a potent molluscicide against B. glabrata, a snail which normally harbors schistosomes. Thus it may be possible to use cashewnut-shell liquid to control the population of fresh water snails and the spreading of schistosomiasis.

2. Plant extracts and pure natural products of pharmacological interest (in collaboration with Dr. G. Kapadia, Howard University). Examination and characterization by GC-MS.

3. Insect pheromones (with Dr. M. S. Blum, U. of Georgia and Dr. A. Hefetz, Tel Aviv University). Study of the exocrine glands extracts of the Israeli weaver ant, Polyrhachis simplex, revealed the presence of an uncommon insect product in the mandibular glands, 4-heptanone, accompanied by 6-methyl-5-hepten-2-one and its corresponding alcohol. These compounds may play a role in the seasonal migrations of the weaver ants.

4. Work on the composition of the anal gland secretions of Herpestes ichneumon (Israeli mongoose, important in the ecology of Israel) was concluded (in collaboration with Dr. A. Hefetz, Tel Aviv University). The secretions are complex mixtures of polymethylsubstituted fatty acids of various chain lengths (C_8 to C_{20}). Only one of the acids is 2-Me substituted, the sex-specific 2,4,6,10-tetramethyl undecanoic acid, found only in the male mongoose.

5. (In collaboration with Dr. D. Lundgren, PM, NIADDK). The study on the identification and quantitation of 2-pyrrolidone isolated from the brain of rats by HPLC was concluded. This method was then applied to follow the metabolism of Δ-pyrroline to 2-pyrrolidone by rat liver. It was shown that Δ-pyrroline is an intermediate metabolite in the pathway from putrescine to 5-hydroxy-2-pyrrolidone.

Publications

1. Lloyd, H. A., Denny, C., and Krishna, G. A simple liquid chromatographic method for analysis and isolation of the unsaturated components of anacardic acid. J. Liquid Chromatog. 3: 1497-1504 (1980).

2. Lundgren, D. W., Lloyd, H. A. and Hawkins, J. Δ-Pyrroline: intermediary metabolite in the conversion of putrescine to 2-pyrrolidone. Biochem. Biophys. Res. Comm. 97, 667-672 (1980).

3. Sullivan, J. T., Richards, C. S., Lloyd, H. A., and Krishna, G. Anacardic acid: Molluscicide in cashew nutshell liquid. Planta Medica (in press).

4. Hefetz, A. and Lloyd, H. A. The exocrine glands of *Polyrhachis simplex* - Chemistry and function. *Chemical Ecology* (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01005-10 CH

PERIOD COVERED

October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)

X-ray Structural R & D for Physiologically Important Molecules

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

PI:	J. V. Silverton	Research Chemist	CH	NHLBI
OTHERS:	A. Bavoso	Visiting Fellow		NHLBI
	C. A. Coulter	Guest Worker		DRR

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemistry

SECTION

Chemical Structure Section

INSTITUTE AND LOCATION

NHLBI-NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.4	1.4	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)

Crystallographic studies providing unique or most rapid solutions to structural problems. Development and use of computer techniques for atomic resolution studies of large molecules.

PHS-6040
(Rev. 2-81)

1. Optical activity (with Dr. H. M. Fales)

The investigation of the crystallization behavior of dimethyl tartrate was continued. The crystal structure of the stable form of the racemate was determined but, despite considerable experimental work, it has not been possible to obtain the metastable form of the racemate as single crystals. It has also not been possible to obtain the reported third ("stable") optically active form despite careful reproduction of the experimental details given by Paterson. This is possibly not too surprising since other workers reported that they could only obtain Patterson's third form if given a seed crystal by Patterson (who unfortunately died 40 years ago).

2. Toxins

a) Considerable preliminary work has been done on crystals of the high molecular weight liver mycotoxin, phomopsin (in collaboration with Dr. Claude Culvenor, CSIRO, Australia). Many crystals from different solvents have been investigated with a view to determining the chemical structure of this economically important toxin. However, all crystals so far obtained, while well formed, only diffracted X-rays at very small angles and must, therefore, be microscopically heterogeneous. Possibilities exist for the preparation of a slightly smaller degradation product or an alkaline earth salt and chemical work will continue.

b) An accurate redetermination of the structure and absolute stereochemistry of the mycotoxin, viridicatum toxin, has been carried out. The work represents a collaboration with Dr. T. Akiyama of the School of Pharmacy, University of Tokyo, Japan. The absolute stereochemistry was determined by the anomalous scattering of oxygen using diffractometer programs developed here. The probability of a correct assignment is 0.9999 on the χ^2 test and it is felt that the results are reliable especially since the stereochemistry proves to be the same as that in tetracycline to which the toxin is related.

3. Chemical problems

a) Five membered rings.

In collaboration with Dr. James Cooke of the University of Wisconsin-Milwaukee, two interesting systems of four fused five-membered rings have been investigated. The aim of the crystallographic work is to increase our understanding of the chemical reactivity of such compounds and also to understand the conformations adopted by these unusual molecules. The lability of two compounds is in a sense opposite to that which might be expected on simplistic grounds and even different from what might be deduced from ring-strain determined from the crystal structure. It is now clear that much more subtle geometric effects are important and semi-theoretical work is continuing.

b) Solid state rotamers of cis-6'-bromo-N-formyl-nor-reticulene (collaboration with Drs. Peter Buchs and Kenner Rice, NIADDK).

There appear to be two rotamers of this compound with a fairly large energy barrier allowing the possibility of isolation of solid specimens of each rotamer. So far only one rotamer has been obtained crystalline and its crystal structure has been determined. It is possible to understand why the rotamer is stable from the X-ray results and also to infer why the other form is difficult to isolate although attempts will be continued to achieve this result.

c) 7-butyl-8-hydroxy-1-azaspiro[5.5]undecane hydrobromide (in collaboration with Drs S. Takahashi and A. Brossi, NIADDK).

This compound is related to the acetylcholine inhibitor dihydroisohistrionotoxin. It has been possible to show that the stereochemistry is very similar to other acetylcholine inhibitors and, if current theories of inhibition are correct, it should behave similarly. There is a possibility that the compound might act as a carrier for bromine ions across membranes but the association of the ions is not extremely strong. The determination of the conformation allows an understanding of the unusual difficulty which was experienced in making the salt.

4. Chemical analysis and stereochemistry.

The structure of the cardiac glycoside, humistratin (collaboration with Drs. K. Nishio and Murray Blum, Department of Entomology, University of Georgia).

The chemical problem here involved the location of a double bond whose position had proven impossible to determine by other physical techniques. The problem was solved successfully and the steroid moeity proves to be new although related in general structure to those found in other cardiac glycosides. In the course of the investigation, ample justification was obtained for the general principle of this laboratory of carrying out crystal structure determinations as accurately as possible. While we were not in any danger of reporting a wrong result, it is interesting that the model being used was incorrect in a minor respect at an R-factor of 5.2%; a level at which many investigators consider the results incontrovertible and eminently publishable. The final R-factor obtained was 3.4% (a fairly standard value for this laboratory).

5. Peptides and nucleotides.

This topic is of especial interest to Dr. Alfonso Bavoso. Many different peptides are being investigated with a view to finding suitable crystals. One crystal which appered very interesting was a putative nucleotide-peptide complex prepared by Dr. Niu of NIADDK. Unfortunately the X-ray work showed that the crystals only contained the peptide, TBOC-(gly)3-benzyl ester.

Dr. Bavoso has obtained a very accurate structure and attempts will continue to attempt to isolate the complex for which there is good evidence for existence in solution.

6. Biochemically interesting metal complexes. (Dr. C. A. Coulter, DRR, and Dr. R. Berger, Laboratory of Chemical Instrumentation, NHLBI)

Dr. Coulter has been investigating the calcium complex of EGTA as a guest worker in the laboratory and has obtained some promising crystals whose crystal structure will be investigated once chemical analysis is complete.

7. Programs and algorithms.

a) Need for modification of diffractometer programs to measure very weak reflections accurately was indicated in our work on the viridicatum toxin. It was also apparent that careful testing to avoid possible large noise signals was needed since the experiments on one crystal reflection take several hours. The modifications have been completed successfully and the programs account for the high apparent reliability of the toxin result.

b) With the advent of the new WYLBUR text editor, work has proceded on preparing "Command procedures" to simplify and expedite data input for the more common techniques use in the laboratory. Presently such procedures have been written for cell dimensions and structure solution programs.

Publications:

1. Scovill, J. P., and Silverton, J. V. An unusually facile ring opening reaction in the pyridine system. J. Org. Chem., 45, 4372-4376, 1980.

2. Frew, J. A., Proctor, G. R. and Silverton, J. V. Rections of the sodium salts of some heterocyclic beta-ketoesters with dimethyl acetylenedicarboxylate. J. Chem. Soc. Perkin I, 1251-1256, 1980.

3. Ungemach, F., Soerens, D. S., Weber, R., DiPierro, M., Campos, O, Mokry, P, Cook, J. M., and Silverton, J. V. General method for assignment of stereochemistry of 1,3-disubstituted 1,2,3,4-tetrahydro-beta-carbolines by carbon-13 spectroscopy. J. Amer. Chem. Soc. 102, 6976-6984, 1980.

4. Rosner, M., Brossi, A., and Silverton, J. V. Structure, chemistry, and antimalarial properties of mefloquine-aziridine. Heterocycles 51, 925-933, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01006-10 CH

PERIOD COVERED

October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)

The Characterization of Natural Materials

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

PI:	R. J. Highet	Research Chemist	CH	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemistry

SECTION

Structural Nuclear Magnetic Resonance Section

INSTITUTE AND LOCATION

NHLBI-NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

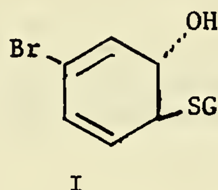
SUMMARY OF WORK (200 words or less - underline keywords)

Metabolism of drugs. The structure of two bile metabolites of bromobenzene have been demonstrated by carbon-13 and J-resolved two dimensional Fourier transform proton nmr spectroscopy.

Streptomyces metabolites. The structure of chrysomycins A and B have been elucidated by carbon-13 and proton nmr spectroscopy.

PHS-6040
(Rev. 2-81)

1. Metabolite studies (with J. A. Ferretti, DCRT; L. Pohl, T. R. Burke, and J. A. Hinson, CP, NHLBI). The structures of two bile metabolites of bromobenzene have been shown by carbon-13 and proton nmr to be I and II. The



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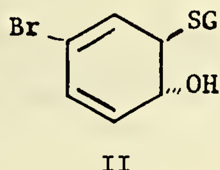
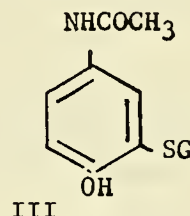
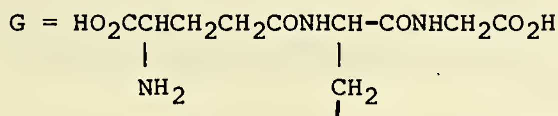


II



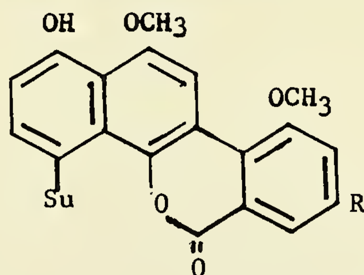
III



possibility of obtaining J-resolved two dimensional Fourier transform spectra at high field was essential to this study. The metabolite of acetaminophen is III.

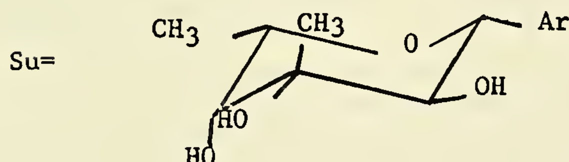
2. Insect pheremones (with M. Blum and T. Jones, Univ. of Georgia). The anal glands of termites produce a substance shown by proton nmr to be 2-hydroxy-6-methylacetophenone.

3. Streptomyces metabolites (with U. Weiss, NIADDK and members of the Frederick Cancer Research Center). A study has been initiated of the structure of the Streptomyces metabolites chrysoniycin A&B, (IV, V) which have shown promising characteristic antitumor screening tests. Their chromophoric moiety is demonstrated by comparison of the carbon spectra with published data. The structure of the unusual branched-chain sugar is inferred from the proton nmr. Stereochemical assignments will require further study.



IV, R=CH=CH$_2$
V R=CH$_3$

Su=



4. Miscellaneous (a) (with P. Mazzochi, University of Maryland). Study of the products of photochemical addition of alkenes to substituted phthalimides by C-13 and proton nmr has demonstrated their structure, and shown that orientation of the addition is controlled by the interactions in the intermediate biradical of the substituents with the radical electron.

(b) Liquid crystal studies (with C. L. Khetrapal, Raman Research Institute, Bangalore, India) Analysis of the proton, nmr spectrum of phenylpropyne in liquid crystal solution yields results consistent with an undistorted benzene ring but insensitive to the rotation of the methyl group.

Publications

1. Highet, R. J., Covey, D. F., and Robinson, C. H. Carbon-13 nuclear magnetic resonance spectra of 5,10-secosteroids. A transannular intramolecular hydrogen bond. J. Org. Chem. 45, 3286-9 (1980).

2. Highet, R. J., Daly, J. W., Fujiwara, T., and Tokuyama, T. Indolizidine alkaloids from poison frogs of Dendrobates spp. Planta Medica, 39, 260-61 (1980).

3. Pohl, L. R., Branchflower, R. V., Highet, R. J., Martin, J. L., Nunn, D. S., Monks, T. J., George, J. W., and Hinson, JH. A. The formation of diglutathionyl dethiocarbonate as a metabolite of chloroform, bromotrichloromethane and carbon tetrachloride. Drug Metab. Disp. in press.

4. Khetrapal, C. L. and Highet, R. J. PMR study of partially oriented phenylpropyne, J. Mag. Res. in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01008-11 CH

PERIOD COVERED
October 1, 1980 - September 30, 1981

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

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	S. R. Heller	Computer Specialist	MIDSD	EPA
	R. Potenzone, Jr.	Chemist	MIDSD	EPA

COOPERATING UNITS (if any)

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LAB/BRANCH
Laboratory of Chemistry

SECTION
Chemical Structure Section

INSTITUTE AND LOCATION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.3	2.3	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)

Components of the system can be categorized as spectroscopic environmental or regulatory. The spectroscopies covered include mass spectrometry, nmr spectroscopy, IR spectroscopy and X-ray diffraction. The environmental data bases deal with chemical spills, acute toxicity, composition of commercial products and environmental disposition. In the regulatory area, data bases of chemicals regulated by and proposed for regulation by the U.S. Government are maintained.

During this reporting period, management of CIS operations has been a prime responsibility. Particular emphasis has been placed upon user documentation, which has been completed, and research into techniques for evaluation of numeric data. A number of interagency agreements and contracts for the continued growth of the CIS have been managed.

PHS-6040
(Rev. 2-81)

Development and operation of the NIH-EPA Chemical Information System is a multi-agency project involving seven agencies of the U. S. Government and over 40 groups in 15 different countries.

Management and coordination of this effort has been carried out during the reporting period. Specifically, this includes: negotiation and maintenance of five interagency agreements, a prime contract for operations support and six subcontracts, primarily for operations support abroad; close collaboration with EPA staff in the design and development of new CIS components; monitoring of the existing system for problems and errors; supervision of the production and publication of User's Manuals, 15 of which are now in print, and a monthly Newsletter, now in its third year, which is mailed to over 2,600 readers.

In the area of development, a study of data evaluation and validation methods is being carried out in collaboration with MBS. Development has begun of new CIS components. These include a searchable file of infrared spectra, a search system for nucleic acid sequences, and a search system for toxicities of commercial products.

<u>Publications</u>:

1. Heller, S. R., and Milne, G. W. A.: The NIH-EPA Chemical Information System. In Koptyugh, V (Ed.) Proceedings of the 4th International Conference on Computers in Chemistry. Novosibirsk, USSR, 1979, pp. 270-302.

2. Heller, S. R., and Milne, G. W. A.: Linking Scientific Data Bases - The NIH/EPA Chemical Information System. Database 3: 45-57, 1980.

3. Heller, S. R. and Milne, G. W. A.: The NIH-EPA Chemical Information System in Support of Structure Elucidation, Proceedings of the 1979 COBAC Conference, Anal. Chimica Acta, 122, 1197-138, 1980.

4. Milne, G. W. A., and Heller, S. R.: The NIH/EPA Chemical Information System, J. Chem. Inf. and Comp. Sci., 20, 204-211, 1980.

5. Heller, S. R., and Milne, G. W. A.: How to Plug Into a Computer Listing of Chemical Information. I. Industrial Chemical News, 1: 20, 1980.

6. Heller, S. R., and Milne, G. W. A.: How to Plug Into a Computer Listing of Chemical Information. II. Industrial Chemical News, 2: 19, 1980.

7. Dillard, J. G., Heller, S. R., McLafferty, F. W., Milne, G. W. A. and Venkataraghavan, R.: Critical Evaluation of Class II and Class III Electron Impact Mass Spectra. Operating Parameters and Reporting Mass Spectra. Org. Mass Spec. 16: 48-49, 1981.

8. Heller, S. R., and Milne, G. W. A.: The NIH-EPA Mass Spectral Handbook. NBS-NSRDS 63, Suppl. #1, pp. 1292). GPO Ordering No. 003-003-02268.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01012-08 CH

PERIOD COVERED
October 1, 1980 to September 30, 1981

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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TOTAL MANYEARS: 2.4	PROFESSIONAL: 1.2	OTHER: 1.2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
Gila monster venom contains a peptide that stimulates pancreatic enzyme secretion probably by a mechanism involving interaction with vasoactive intestinal peptide receptors on pancreatic acinar cells. Four peptides have been isolated from venom sacs of two species of yellow jackets and their amino acid composition determined. Three of the peptides lyse liposomes indicating that they will cause mast cell degranulation. The European hornet contains a peptide which depolarizes human neutrophil membranes without causing cell death. The peptide also causes the cells to secrete their granular contents suggesting a pro-inflammatory activity. Of no less interest, the peptide blocks chemoattractant-induced secretion of the cells. Metenkephalin blocks the action of ranatensin on the guinea pig ileum probably at the level of the ranatensin receptor. Numerous columns and mobile phases have been evaluated for the HPLC analysis of peptides. Best results (resolution, recovery, sensitivity) were obtained with Toyo Soda TSK LS-410 columns and a mobile phase consisting of trifluoracetic acid, triethylamine, water, and acetonitrile. With many peptides as little as 0.2 μg could be detected.

PHS-6040
(Rev. 2-81)

Objectives:

1. Isolate, characterize and determine the significance of new biologically active peptides from natural sources.

2. Develop more effective methods for the microanalysis of peptides.

Methods: High-performance liquid chromatography (HPLC) and other chromatographic techniques, bioassay, antibody production and evaluation.

Major Findings:

1. New Biologically Active Peptides: We are continuing to examine venoms in the search for new biologically active peptides. In general, methanol extracts of venoms were chromatographed on Sephadex G-25 columns and active fractions further purified by HPLC. Amino acid analyses were performed on active fractions corresponding to discrete UV peaks.

Gila Monster. Methanol extracts of Gila monster venoms stimulated amylase secretion of guinea pig pancreatic acinar cells. The venom extract increased enzyme secretion to the same extent as did VIP, secretin, and 8-bromo cyclic AMP. The venom also potentiated the release of amylase caused by secretagogues whose actions are mediated by their ability to mobilize cellular calcium, (e.g., CCK, acetylcholine, and bombesin). The venom did not, however, alter the increase in enzyme secretion caused by secretagogues whose actions are mediated by cyclic AMP (e.g., VIP and secretin). The venom caused a 30-fold increase in cellular cyclic AMP and inhibited the binding of ^{125}I-VIP to its membrane receptors in pancreatic acinar cells. The actions of the venom on enzyme secretion and on cyclic AMP were inhibited by VIP receptor antagonists (e.g., VIP 10-28 and secretin 5-27) but were not affected by a cholinergic receptor antagonist (atropine) or a CCK receptor antagonist (dibutyryl C-GMP). No immunoreactive VIP could be detected in the venom by radioimmunoassay. Incubation of the venom with chymotrypsin, pronase, and thermolysin destroyed the secretagogue activity. These results suggest that Gila monster venom contains a peptide that may stimulate pancreatic enzyme secretion by interacting with VIP receptors on pancreatic acinar cells, thereby activating adenylate cyclase and increasing cellular cyclic AMP.

Unfortunately, attempts so far to purify the pancreatic secretagogue from Gila monster using conventional and high performance liquid chromatography have been unsuccessful. Aliquots of the 80% methanolic and 1% acetic acid extracts were chromatographed on Sephadex G-25, Biogel P-4, Sephadex C-25 and HPLC columns. In each case, activity was found to be smeared over many fractions. The activity could not be assigned to a discrete peak.

Vespula germanica (yellow jacket). Three peptides (VG I, VG II and VG III) have been isolated in pilot studies.

Peptide	Amino Acid Composition (residues/mol)								
VG I	Asp 1.10	Ser 0.91	Glu 1.49	Gly 1.27	Ala 2.00	Ile 2.48	Leu 4.08	Phe 2.10	Arg 0.98
VG II	Asp 0.88	Ala 3.00	Ile 2.05	Leu 3.60	Lys 2.07				
VG III	Gly 1.94	Ala 1.00	Ile 1.30	Leu 4.28	Phe 0.95	Lys 1.08			

Peptides VG II and VG III, but not VG I, stimulated the intracellular-release of 6-carboxy fluorescein from liposomes. This indicates that they are surface active and cause mast cell degranulation like mastoporan which they resemble. Another mastoporan-like peptide VM I has been detected in Vespula maculifrons (yellow jacket).

	Gly	Val	Ile	Leu	Phe	Lys	Asn	Ala
VM I	1.35	1.00	0.95	4.36	1.01	2.04		
Mastoporan			2	4		3	1	4

Vespa Crabro (European hornet). Fractions were assayed for inhibition of F-Met-Leu-Phe-induced activation of human neutrophils. A single peak of activity was obtained. The activity was destroyed by hot 6 N HCl and by incubation with chymotrypsin, trypsin, and pronase.

Another aliquot of the most active fraction from the Sephadex G-25 column was analyzed by HPLC and fractions collected for bioassay. A peak of activity which corresponded to the major UV absorbing peak has been obtained.

The active peptide is of considerable interest because it causes membrane depolarization without cell death. Furthermore, it causes the cells to secrete their glandular content suggesting a pro-inflammatory activity. In addition to membrane depolarizing and secretory activity, the peptide also blocks chemoattractant-induced membrane polarization and secretion of the cells. The peptide appears to be equipotent as an inhibitor of chemoattractants and membrane-depolarizing agent.

Ranatensin. The antagonistic action of metenkephalin against ranatensin on the guinea pig ileum has been determined over the entire dose-response range of ranatensin. Metenkephalin causes a parallel displacement in the dose-response curve indicating that it exerts its effect at the level of the ranatensin receptor.

Antibodies. Burro 309 antibody to the N-terminus of bradykinin can be used to measure functional high molecular weight kininogen in plasma. Plasma (collected in ACD with o-phenanthroline-EDTA, treated with polybrene and NaN_3) is treated with human plasma kallikrein, and the liberated kinin is measured by RIA. Treated plasmas contain 700-1,000 ng immunoreactive kinin/ml, compared with 30-45 ng/ml in untreated plasma, and are parallel to the standard curve over the usable range of the assay. Reagents used to treat plasmas do not interfere in the assay in these concentrations.

Bound counts can be separated from free counts either by $(NH_4)_2SO_4$ precipitation or Millipore filtering. Both methods are touchy - presumably because of the low avidity of the antibody. In a typical assay using a final antibody dilution of 1:2,800 and 13,000 cpm of tracer, the ED_{50} is ∿130 pg, and the best range of the assay (<10%CV) is 33-1,100 pg.

Cross-reactivity of the antibody is: >87% with des-arg-9 bradykinin, >37% with des-phe-8-arg-9 bradykinin, 0% with B_4 kininogen, <6% with a sample of B_2 kininogen (purified by HPLC, from which 158 ng/ml kinin could be released by

trypsin incubation. Polisteskinin, des-arg-1 bradykinin, lys-bradykinin, met-lys-bradykinin, and two other kininogen samples do not cross-react.

Attempts to boost the titer of burro 309 have not been successful. Purification of the antibody with DEAE Sephadex A-150 and $(NH_4)_2SO_4$ precipitation yielded 1/3 of the original activity. No activity was recovered from an attempted purification on Protein A-Sepharose.

Antibodies to ranatensin (max. 69% binding at 1:800); and bombesin (max. 34% at 1:400) are forming in rabbits.

2. HPLC Analysis of Amino Acid Phenylthiohydantoins. In order to provide a more easily reproduced HPLC method than those previously published, a procedure employing stepwise, instead of gradient, elution has been developed. Parameter limits are presented along with specific recommendations for the resolution of potentially troublesome derivatives. Employing a single-step change of mobile phases, all the common PTH's are identified in a single 12 min run on a 15-cm Zorbax ODS column and a 14 min run on a 25-cm column. Conditions for the 15-cm column-initial mobile phase, 0.035 M sodium acetate, pH 5.0: CH_3CN, 85: 15(^{v}/v); final mobile phase, 58:42; switching time, 5.0 min; inlet pressure 435 psi; flow rate 1.0 ml/min; temperature 62°C. Conditions for the 25-cm column - 0.025 M sodium acetate, pH 5.1: CH_3CN, 85:15 (^{v}/v); final mobile phase, 59:41; switching time, 6.0 min; inlet pressure 1550 psi; flow rate 1.5 ml/min; temperature 63°C.

3. HPLC Analysis of Peptides. Since HPLC is an important technique we employ for the isolation and characterization of new biologically active peptides, we have compared ten different columns from several manufacturers using five different mobile phases and five peptide mixtures for the purpose of determining the combination which gives the best resolution, peak shape, sensitivity and recovery. Columns tested were all 0.46 x 25 cm packed with 5 or 10 micron particles and included: TSK LS-410, μ Bondapak, Zorbax ODS, Zorbax ODS 150 Å, endcapped, Zorbax C-8, Zorbax C-8 150 Å, endcapped, Zorbax CN, Zorbax TMS, Supelco C-18, and Supelco C-1.

Mobile Phases

a. Solution A = 0.05% TFA - 0.025% TEA (pH 2.5)
Solution B = 0.05% TFA - 0.025% TEA - 80% ACN
Gradient = 30% B → 100% B, 21 min

b. Solution A = 0.6 M $NaClO_4$ - 0.1% H_3PO_4 (pH 1.9)
Solution B = 0.1% H_3PO_4 in ACN
Gradient = 30% B → 100% B, 21 min

c. Solution A = 0.1 M $NaClO_4$ - 0.1% H (pH 2.0)
Solution B = 0.1% H_3PO_4 in ACN
Gradient = 30% B → 100% B, 21 min

d. Solution A = 0.25 N H_3PO_4 (pH 2.1 with TEA)
Solution B = 0.25 N H_3PO_4 in ACN
Gradient = 10% B → 100% B, 45 min

e. Solution A = 0.25 N H_3PO_4 - 0.02% NaN_3 (pH 3.1 with TEA)
   Solution B = ACN
   Gradient = 0% B → 100% B, 60 min

Peptide Mixtures

(M-7)	(M-8)	(M-9)
α-Endorphin	Bradykinin Potentiator B	Lys-Bradykinin
Met-Enkephalin	Lys-Bradykinin	Bradykinin
Lys-Enkephalin	Met-Lys-Bradykinin	Ranatensin
Bombesin	Ranatensin	Substance P
Ranatensin	Insulin B Chain	Neurotensin
Angiotensin I		
Angiotensin II		
Angiotensin III		
Mastoporan		

(M-11)	(M-12)
Cytochrome C	Glucagon
Insulin B Chain	Insulin
Lactalbumin	Melittin
Ovalmin	Albumin
Myoglobin	

Best results were obtained with mobile phase (a) and TSK LS-410 columns. The μ Bondapak column was rated second. Of all mobile phases, (a) gave the best results with all columns tested. Peptide mixtures M-7, M-8, and M-9 were satisfactorily analyzed on all columns but M-12 and especially M-11 adsorbed to varying degrees to all columns except the two mentioned above. Components of M-11 either adsorbed completely or only partially eluted. Those which partially eluted could be further eluted by repeating the solvent gradient up to four times. The peaks decreased each time. With our best system (TSK LS-410 column, mobile phase (a) this phenomenon was not observed. However, in tests with ribonuclease injections of less than 0.2 μg give no peaks. Other tests with bradykinin, glucagon and ranatensin were, on the other hand, very encouraging. A line through the origin was obtained when peak height was plotted against amount injected down to 100 ng.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01016-11 CH

PERIOD COVERED

October 1, 1980 to September 30, 1981

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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LAB/BRANCH

Laboratory of Chemistry

SECTION

Section on Physiological Chemistry

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.6	2.6	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

1. Rat renal lymph contains immunoreactive glandular kallikrein which does not appear to be biologically active. Lymph also contains high levels of renin and low levels of angiotensin converting enzyme which appear to be the only kininase in lymph. In addition, pooled lymph contains very high levels of trypsin inhibitor(s) and lower levels of kallikrein inhibitors.

2. Rat renal kallikrein is present in high levels in the outer cortex and decreases from outer cortex to inner medulla.

3. Whole human saliva contains kininogen antigens. Levels in parotid saliva are much lower.

4. Parotid salivary kallikrein is elevated in patients with aldosterone-producing tumors and returns to normal upon removal of the tumor.

PHS-6040
(Rev. 2-81)

Objectives: To improve procedures for the assay of components of the kallikrein-kinin system and to use these procedures to establish the role of the system in health and disease.

Methods: Bioassay, radioimmunoassay, radiochemical and chromogenic substrate assays, chromatographic and electrophoretic techniques, immunochemical procedures.

Major Findings:

Rat Renal Lymph: Rat renal lymph contains immunoreactive glandular kallikrein which cross-reacts in a parallel manner in an RIA for rat urinary kallikrein (RUK). Measurement of 14 individual lymph samples gave a value of 260 ± 12 ng antigen/ml ($\bar{x}$ ± SEM) and recovery of kallikrein added to lymph was 92 ± 10% (n=4). Pooled lymph from 7 additional male Sprague-Dawley rats, containing 400 ng antigen/ml, had both TAME esterase and S2266 activity but contained no kininogenase activity, verifying findings with a previous lymph pool. When 25 μl of pooled lymph was incubated with 500 ng kinin for 15 mins at 37°C and pH 7.4, 100 ng of kinin was destroyed. All of this kininase activity is due to angiotensin converting enzyme as it can be completely inhibited by Captopril or EDTA. Renal lymph contains ~100-fold the renin levels of normal rat plasma but has substantially less converting enzyme since, while lymph could generate angiotensin II from angiotensin I, it had no detectable activity against Hip-Gly-Gly, a converting enzyme substrate commonly used for plasma assay. Enzyme inhibitor assays using S2266 showed that, while pooled renal lymph contains less kallikrein inhibitor than normal rat plasma, it has higher levels of trypsin inhibitory activity. An attempt to remove these inhibitors using immobilized anhydrotrypsin failed.

Rat Renal Kallikrein: Preparation of a new, highly purified RUK standard has significantly improved the RIA for this enzyme and the new RUK is being used to raise antibodies in rabbits with the hope of producing an antibody to the active site. In order to study kallikrein in the kidney a range of detergents were tested for their effects on the assay. Sodium thiocyanate and sodium deoxycholate had very severe effects on the assay but two zwitterionic propanesulfonate detergents with 12 and 14 carbon chain lengths (N-12 and N-14) had minimal effects and Triton X-100 had no effect. Comparison of the kidney homogenates produced with N-12, N-14 and Triton X-100 with a control homogenate showed that all three detergents were effective at solubilizing renal kallikrein. Since identical values were obtained with all three detergents, it was assumed that this represented maximum solubilization. Triton X-100 was used for all further work. Homogenates of detergent-treated kidneys contained three-fold the levels of kallikrein in the control homogenates. A level of 3.5 μg kallikrein/g tissue suggest that the kallikrein content of both kidneys is only about 10-15% of the daily urinary output. Homogenates of whole kidney or separate cortex and medulla preparations were all parallel to the standard and recovery of added kallikrein was around 100% in all preparations. Measurement of kallikrein in kidney slices showed that the enzyme

decreases from outer cortex to inner medulla with values showing good reproducibility from animal to animal. Studies are underway to show the effects of restriction of dietary sodium on the levels and distribution of killikrein in kidney slices. The collagenase treatment of kidney used in dissection of tubules has no effect on the assay and preliminary experiments suggest that measurement of kallikrein in isolated tubules should be possible.

Kinin Radioimmunoassay: The previously described (Project No. Z01 H2 01012-07CH) RIA based on an antibody to the N-terminus of bradykinin has been more firmly established and characterized. It was previously shown that the antibody showed no cross-reactivity with polisteskinin, des-arg-1-bradykinin, lys-bradykinin, met-lys-bradykinin and two partially purified kininogen samples. In addition it is now known that there is no cross-reaction with highly purified HMW or LMW kininogens. Substantial cross-reaction, however, is seen with the bradykinin metabolites des-arg-9 bradykinin (>87%) and des-phe-8-arg-9 bradykinin (>37%). The assay has also been used as a measure of functional HMW kininogen by measuring kinin release following treatment with purified human plasma kallikrein. Treated plasmas are parallel to the standard curve and contain 700-1000 ng immunoreactive kinin/ml, which is good agreement with typical bioassay values. In comparison, untreated plasma contains 30-45 ng antigen/ml which may, in part, represent kinin metabolites (as other assays have shown that plasma contains less than 1 ng kinin/ml).

Salivary and Renal Kininogens: Parotid and whole human saliva samples were assayed for HMW and total (HMW and LMW) kininogens by RIA. Parotid salivas (n=10) contained low levels of total kininogen (6.5 ± 0.8 ng/ml, $\bar{x}$ ± SEM) and little or no detectable HMW kininogen. Whole salivas, however, had clearly measureable levels of both antigens and cross-reacted in a parallel manner in both assays. Measurement of nine samples revealed 45 ± 14 ng HMW kininogen/ml ($\bar{x}$ ± SEM) and 120 ± 29 ng total kininogen/ml. In contrast to the results for kininogen, the kallikrein concentrations in parotid and whole salivas are very similar.

Attempts were made to quantitate both total and HMW kininogens in monkey kidney cortex and medulla but problems occurred. The kidney preparations did not show parallelism on either RIA. Attempts to partially purify the kininogens by immunoaffinity chromatography had mixed results since leakage of antibody from the gel prevented meaningful RIA measurements.

Glandular Kallikreins: Clinical studies involving the measurements of salivary and urinary kallikrein have been concluded and methodology and problems in measurement of urinary kallikrein have been studied. Salivary kallikrein increases in response to salt restriction or Fludrocortisone and abnormally high levels are seen in patients with aldosterone-producing tumors. Values decrease to normal in these patients following removal of the tumor. In patients with minimal thyroid function, urinary assays reveal that there is an impaired ability to activate prokallikrein as there is little active enzyme but normal levels of the proenzyme.

The RIA measures both free and prokallikrein. This was demonstrated by showing cross-reactivity of purified prokallikrein and by showing no change in the antigenic contents of urine following trypsin activation of endogenous prokallikrein. Also in some patients possessing normal levels of prokallikrein but negligible free, antigenic activity was still seen. A correlation study of RIA vs. free esterase and RIA vs. Total esterase was performed on 113 urine samples. For RIA vs. free

esterase (r = 0.73) a correlation line described by $y = 18.4x + 106.7$ was obtained while for RIA vs. total esterase (r = 0.87) the line obeyed the relationship $y = 16.7x - 6.2$. Once again this is a demonstration that the RIA measures both free and prokallikrein. In addition the intercepts suggest that there is very little non-kallikrein esterase in urine. This was demonstrated further by batch adsorption of 17 individual urines with immobilized antibody to HUK. In comparison to controls the antibody removed 84 ± 1.1% of the urinary esterase.

A major problem in the measurement of urinary kallikrein relates to stability. Pure HUK is stable indefinitely at -20°C but in many urines stored in this manner there is a rapid decrease in both esterase and RIA activity compared to samples stored at 4°C. Losses were more severe in samples which had been collected under toluene and then frozen. Storage at 4°C is also not ideal since there is a more rapid conversion of prokallikrein to the active enzyme under these conditions compared to frozen samples. One additional problem observed with esterase measurements was the loss of kallikrein during gel filtration to remove salt. These losses appear to be inversely related to the protein content of the sample.

Proposed Course: Renal lymph studies will be continued to gain more definitive measurements on inhibitor levels and content of angiotensin converting enzyme. Lymph will also be examined for the presence of kininogen. Work on rat renal kallikrein will be continued to examine the effects of salt restriction on the level and distribution of the enzyme. Attempts will also be made to quantitate kallikrein in isolated tubular segments. The kinin RIA will be applied to the measurement of functional kininogen in samples from pregnant women and may be used to look for kinin metabolites. Some further experiments will also be performed to determine the best method to measure prokallikrein and free kallikrein in human urine.

Publications:

1. Lawton, W.J., Proud, D., Frech, M.E., Pierce, J.V., Keiser, H.R., and Pisano, J.J.: Characterization and origin of immunoreactive glandular kallikrein in rat plasma. Biochem. Pharmacol. 30:1731-1737, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01018-24 CH

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Biochemistry of the Kallikrein-Kininogen-Kinin System

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

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COOPERATING UNITS (if any)

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CHECK 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 renal kallikrein and prokallikrein have been purified and separated by immunoaffinity chromatography; affinity chromatography on immobilized basic pancreatic trypsin inhibitor (Trasylol), lily bulb trypsin inhibitor, and Concanavalin A; gel filtration; and hydroxyapatite chromatography. The proenzyme after trypsin activation has a specific activity of 19 TAMe units (TU)/A_{280}, while our purest active enzyme has a specific activity of 45 TU/A_{280}. Evidence is presented that pure active enzyme will have a specific activity of 59-60 TU/A_{280}.

A potent new inhibitor of human polymorphonuclear (PMN) leukocyte elastase has been obtained from calla flower bulb extracts. The only partially purified calla bulb inhibitor (M_r=33,000 and pI=5.3) is a much better inhibitor of this elastase than previously reported trypsin inhibitors from soy beans, lima beans, and pancreas, and also does not inhibit pancreatic elastase.

PHS-6040
(Rev. 2-81)

Objectives: Purification of glandular kallikreins and prokallikreins and of components of the plasma kinin, clotting, and fibrinolytic systems for purposes of characterization and production of specific antiserums. Preparation of purified specific antibodies and isolation from plant and animal sources of specific inhibitors for human plasma and glandular proteases (kallikreins, Hageman factor, plasmin, thrombin, Factor XI, elastases, etc.) for biochemical, clinical, and other studies. Preparation of affinity adsorbents from purified antibodies, antigens, enzymes, and inhibitors for purification and other purposes, such as devising specific biochemical and radioimmunochemical assays. Application of these purified materials, affinity adsorbents, and assay methods to studies of normal and pathological states in man and other primates.

Major Findings:

I. Human Renal Kallikrein and Prokallikrein.

A. Prokallikrein (see Z01 HL 01018-21 LC). The following steps were used at 4-6° to purify prokallikrein from human urine: (1) 100-fold concentration of urine containing ∿ 0.02% NaN_3 with an Amicon hollow-fiber apparatus, removal of Tamm-Horsfall glycoprotein (THG) by 0.4 M NaCl precipitation, and further hollow-fiber concentration of the supernate to 1,000- to 3,000-fold relative to the starting urine; (2) immunoaffinity chromatography on a column of immobilized antibody to human renal kallikrein and elution of the washed column with 8 M guanidinium chloride (Gdm.Cl); (3) Sephadex G-25 gel filtration of the eluate, concentrated by ultrafiltration, to remove Gdm.Cl; (4) batch affinity adsorption on BPTI (basic pancreatic trypsin inhibitor; Trasylol) -Affi-Gel 10 to remove renal kallikrein; (5) batch affinity adsorption to immobilized monospecific and purified antibody to THG to remove the low molecular weight THG which co-purifies with renal kallikrein; (6) repeat affinity adsorption with BPTI-Affi-Gel 10 to remove renal kallikrein which had formed from the proenzyme during the previous step and storage; and (7) concentration to a small volume with a Millepore immersible CX ultrafiltration unit. After Step 6, the yield of prokallikrein from the starting urine concentrate was 80% with a 990-fold purification. However, after Step 7, these values dropped to 47% and 730-fold, respectively. The specific activity of Step 6 prokallikrein after trypsin activation was 19 TU/A_{280}. When Step 5 prokallikrein (12 TU/A_{280}) was rechromatographed on an anti-kallikrein-agarose column, about 25% of the protein (A_{280}) was not adsorbed. Immunization of a sheep with macerated sections, containing 0.04 A_{280} units (AU) of protein, from polyacrylamide disc gel electrophoresis of the prokallikrein fraction (18 TU/A_{280} after trypsin activation) described previously (Z01 HL 01018-21 LC) and boosting with similar sections containing 0.06 AU of protein on Day 29 gave no detectable precipitating antibody in Ouchterlony analysis. However, a boost on Day 78 of 0.3 AU of Step 7 prokallikrein gave a weak precipitin line on Day 106, while a further boost of 0.22 AU of the same fraction gave a strong line on Day 114. This antiserum appears to be monospecific.

B. Kallikrein. At least the first two steps of the above purification scheme for renal prokallikrein are used also to purify the active enzyme. Methods for further purification were explored, some of which can probably be applied to prokallikrein purification.

1. Hydroxyapathite chromatography of Step 2 kallikrein. The 8 M (final concentration, 2.5 M) Gdm. Cl eluate (0.5 A_{280}; ∿ 10 TU/A_{280}) from the immunoaffinity chromatography of a urine concentrate containing only active kallikrein was applied at pH 5.0 and 5° to a small hydroxyapatite column until it was saturated with kallikrein antigen as detected by Ouchterlony analysis. The column had a capacity of ∿ 25 TU/ml of bed volume. After washing the column with 2.5 M Gdm.Cl, water, and 3 M NaCl/0.01 M NaP_i, pH 6.0, the adsorbed kallikrein was eluted stepwise with 3 M NaCl containing 0.1, 0.2, and 0.5 M NaP_i, pH 6.0. About two-thirds of the eluted kallikrein had a specific activity of 20 TU/A_{280}.

2. Sephacryl S-200 gel filtration. Step 3 kallikrein (19 AU in 1.0 ml; 13.4 TU/A_{280}) filtered through a 1.0 X 116-cm column of Sephacryl S-200 gave two nearly separated, sharp protein peaks. The first peak contained nearly all of the THG contaminating the starting sample but very little esterase activity, although considerable kallikrein antigen was detected. The second peak contained ∿ 99% of the recovered esterase activity (97% yield from the starting sample), with an average specific activity of 26 TU/A_{280} (the two peak fractions, containing 80% of the activity, had specific activities of 28 and 30 TU/A_{280}). A portion of the pooled second peak (0.95 AU; 19.7 TU; 20.8 TU/A_{280}) was adsorbed to a slight excess of BPTI-Affi-Gel 10 (20 mg BPTI/ml of bed volume). The filtrate contained 0.62 AU and 0.26 TU. Thus, by difference the adsorbed kallikrein should have 0.37 AU and 19.4 TU, or a specific activity of 59 TU/A_{280}. However, elution with 1.0 M benzamidine.HCl gave only 11 TU, and concentration of the benzamidine eluate with the Millipore device gave only 5.7 TU and 0.26 AU, and thus a specific activity of 21.9 TU/A_{280}.

3. BPTI-Affi-Gel 10 affinity adsorption. Attempts to purify kallikrein by affinity adsorption to immobilized BPTI have given variable results. One of the best preparations (43 TU/A_{280}; 50% yield; 3.2-fold purification) was obtained by adsorbing Step 3 kallikrein (13.6 TU/A_{280}) to an adsorbent having 7.6 mg of BPTI/ml of bed volume and eluting with 0.1 M HOAc/0.5 M KCl/1.0% PEG 6000 pH 4.0. However, one-third of the esterase activity was lost upon concentration with the Millipore device, with concomitant decrease of specific activity to 39 TU/A_{280}. With the adsorbent having 20 mg of BPTI/ml of bed volume, the recovery of kallikrein esterase activity was only 10-20% with specific activities of 15-20 TU/A_{280}. The use of either acidic buffers or benzamidine gave similar results. Also, buffers of lower pH were required to elute esterase activity from the more highly substituted adsorbent.

4. Lily bulb inhibitor-Affi-Gel 10 affinity chromatography. Although we had previously stated that the lily inhibitor isolated from trypsin-agarose columns was less effective in inhibiting renal kallikrein, relative to inhibitory activity for trypsin, than the lily inhibitor prepared from anhydrotrypsin-agarose columns (Z01 HL 01018-23 CH), we later found no such differences when longer preincubation times were used. Therefore, the lily inhibitor purified by trypsin-agarose affinity chromatography was immobilized on Affi-Gel 10 and the resultant affinity adsorbent was used with Step 3 kallikrein (12.6 TU/A_{280}). The adsorbed kallikrein was eluted between pH 4 and 2. The fractions with highest specific activity, 32-39 TU/A_{280}, recovered in 30% yield were the pH

4.0 and 3.5 eluates. The overall recovery of activity (filtrate + eluates) was 69%.

5. Concanavalin A-Sepharose 4B affinity chromatography. When Step 3 kallikrein (14 TU/A_{280}) was chromatographed on a column of Con A-Sepharose, ∿ 50% of the esterase activity was not adsorbed, while the other 50% was adsorbed and could be eluted with 0.3 M D-glucose. Upon rechromatography of the unadsorbed kallikrein, 86% was not adsorbed and the adsorbed 14% could be eluted with D-glucose. On rechromatography of the glucose-free adsorbed kallikrein fraction, only 0.3% of the esterase activity was not adsorbed, while 99.7% of the activity was recovered by eluting with 0.1 M α-methyl-D-glucoside and 0.1 M α-methyl-D-mannoside.

II. New inhibitor of human polymorphonuclear (PMN) leukocyte elastase from calla flower bulbs. Of the nine plants tested for inhibition of PMN leukocyte and porcine pancreatic elastase (see publication 3 below), only the partially purified trypsin inhibitor from calla bulb extracts showed a high level of activity with PMN elastase. The inhibitor, partially purified by Sephadex G-75 gel filtration, had an M_r of ∿ 33,000 and a pI of ∿ 5.3 by isoelectric focusing. The specific activities of this inhibitor preparation were 0.52, 0.76, 1.4, 0.13, and 0.039 inhibitor units (IU)/mg of trypsin, human plasma kallikrein, human plasmin, bovine α-chymotrypsin, and PMN elastase, respectively. No inhibition of pancreatic elastase was observed. A comparison of the specific activity of the still inhomogeneous preparation of calla bulb inhibitor with PMN elastase (0.039 IU/mg) and the specific activities reported for other, more highly purified inhibitors of this enzyme (soy bean trypsin inhibitor: 0.01 IU/mg; lima bean trypsin inhibitor: 0.023 IU/mg; and BPTI: 0.026 IU/mg) suggests that the potency of the pure inhibitor with PMN elastase will be even greater.

Proposed Course of Project: Human Renal Kallikrein and Prokallikrein. Further purification of renal kallikrein and prokallikrein will be attempted by isoelectric focusing, hydroxyapatite gradient elution chromatography, and affinity chromatography. An activation peptide from the proenzyme will be sought after activation by trypsin or other suitable enzymes. Antibodies specific for the active enzyme and proenzyme will be sought for purification and assay purposes.

Publications:

1. Corthorn, J., Imanari, T., Yoshida, H., Kaize, T., Pierce, J.V., and Pisano, J.J.: Isolation of prokallikrein from human urine. Adv. Exp. Med. Biol. 95: 563-574, 1980.

2. Hojima, Y., Tankersley, D.L., Miller-Andersson, M., Pierce, J.V., and Pisano, J.J.: Enzymatic properties of human Hageman factor fragment with plasma prekallikrein and synthetic substrates. Thromb. Res. 18: 417-430, 1980.

3. Hojima, Y., Pierce, J.V., and Pisano, J.J.: Hageman factor fragment inhibitor in corn seeds: purification and characterization. Thromb. Res. 20: 149-162, 1980.

4. Hojima, Y., Pierce, J.V., and Pisano, J.J.: Plant inhibitors of serine proteinases: Hageman factor fragment, kallikreins, plasmin, thrombin, Factor X_a, trypsin, and chymotrypsin. Thromb. Res. 20: 163-171, 1980.

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

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01025-1 CH

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Hypotensive Action of Hageman Factor

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 mechanism of the vasodepressor response to Plasma Protein Fraction (PPF) was evaluated in rats through the use of a specific inhibitor of Hageman factor fragments (HF_f) previously isolated in our laboratory. The hypotensive effect of intravenous injections of the implicated lots of PPF and purified HF_f was blocked by the pre-incubation with corn Hageman factor inhibitor (CHFI). The hypotensive action of glandular kallikrein and bradykinin was not affected by the inhibitor.

The Brown Norway rat has been reported to be deficient in plasma kallikrein and to have diminished plasma kininogen. We attempted to verify these findings. Our data provide no evidence to support these results.

PHS-6040
(Rev. 2-81)

Objectives: The purpose of these studies was to better define the mechanism by which certain lots of PPF lowered blood pressure, and to better document the hypotensive action of HF_f. In addition, we desired to prove the efficacy of CHFI in vivo tests.

Studies involving the Brown Norway rat were conducted to ascertain if this strain would provide an animal model deficient in kallikrein and low in kininogen. The importance of such an animal model in circulatory and inflammation research would be significant.

Methods: Male Sprague-Dawley (200-325 gm) rats were anesthetized with sodium pentobarbital (5mg/kg I.P.). The left femoral artery and vein were cannulated with PE50 tubing, and the arterial cannular advanced to the bifurcation of the aorta. Arterial blood pressure was monitored continuously. All animals were allowed thirty minutes of stabilization prior to the injection of test materials. In studies involving the CHFI, the animals were pre-treated with the angiotensin converting inhibitor SQ14225 five minutes preceeding test injections. Test samples were injected over thirty seconds.

In experiments involving the CHFI, each animal received only one test injection. This was done to prevent the possible role of substrate depletion in the blood pressure response. In the Brown Norway comparison studies, two injections were used per animal in order to determine if substrate (kininogen) levels were different between the two groups.

Statistical analysis was by an Analysis of Variance for Repeated Measures.

Results:

Table I

Corn Hageman Factor Inhibitor Blocks the Hypotensive Action of Plasma Protein Fraction and Active Hageman Factor in Sprague-Dawley Rats

Sample	Mean Arterial Blood Pressure, Torr* Control	Sample**	n	Significance
Ref. 1	109 ± 16.6	62 ± 12.0	4	$p < .05$
Ref. 1 + CHFI	93 ± 13.6	97 ± 15.1	4	$p > .05$
Sample A	85 ± 15.1	42 ± 0.8	2	
Sample A + CHFI	62 ± 0.8	65 ± 8.4	2	
Sample B	66 ± 7.6	30 ± 3.9	2	
Sample B + CHFI	79 ± 7.6	68 ± 0.3	2	
HF_f	101 ± 10.3	42 ± 4.8	4	$p < .05$
HF_f + CHFI	77 ± 13.6	74 ± 13.2	4	$p > .05$
Sample C	88 ± 8.3	88 ± 5.6	3	$p > .05$
Sample C + HF_f	81 ± 2.7	44 ± 2.9	4	$p < .05$
Sample C + HF_f + CHFI	83 ± 4.9	70 ± 5.6	4	$p < .05$

*Mean ± SE; **Samples A and B but not C are lots of Plasma Protein Fraction known to be hypotensive in man and they were withdrawn from the market.

Table II

There was no significant difference in either the blood pressure response or duration of hypotension between Sprague-Dawley (SD) and Brown Norway (BN) rats with either dextran sulfate or HF_f.

Dextran Sulfate 0.56 mg/ml (two injection per animal)

Injection	Blood Pressure torr, mean ± SD control	post	Strain	n
1	86.55 ± 8.9	43.65 ± 2.8	SD	4
2	103.7 ± 13.7	59.51 ± 37.5	SD	4
1	92.01 ± 7.4	39.1 ± 2.1	BN	4
2	97.45 ± 6.2	39.3 ± 3.8	BN	4
Hageman factor fragments (HF_f) 0.077 ug (one injection per animal)				
1	82.26 ± 8	46.25 + 9	SD	5
1	78.24 ± 11.4	44.57 ± 7	BN	5

Duration of Hypotensive Episode (same animals as noted above)

Injection	seconds (mean ± SD)	Material	Strain
1	311 ±111.5	dextran sulfate	SD
1	261.7 ± 76.8	dextran sulfate	BN
2	88 ± 25.5	dextran sulfate	SD
2	118.5 ± 56.7	dextran sulfate	BN
1	204.8 ± 97.1	HF_f	BN
1	248 ± 80.7	HF_f	SD

Preliminary assays for kallikrein activity in Brown Norway and Sprague-Dawley rat plasma reveal no differences.

Significance of Findings: This work clarifies the mechanism of action of PPF on blood pressure and shows that HF_f is a potent hypotensive agent as well as a procoagulant and an inflammatory agent. The efficacy of the new specific CHFI should allow more definitive studies on the pathophysiologic significance of Hageman factor.

The Brown Norway rat is not, as reported by others, deficient in Hageman factor, prekallikrein, or kininogen and is not uniquely suited to the study of the pathophysiologic role of these proteins.

Annual Report of the
Clinical Hematology Branch
National Heart, Lung, and Blood Institute
October 1, 1980 to September 30, 1981

The research of this Branch is directed toward understanding the underlying causes and developing effective treatment for the major red cell disorders. Red cell diseases which produce significant morbidity and mortality include thalassemia, severe hemoglobinopathies of which sickle cell anemia is the most common, and the various syndromes of bone marrow failure. In our laboratory investigations, we attempt to focus the most advanced techniques and conceptual knowledge on several problems. These include analysis of the differentiation and maturation of erythroid stem cells, elucidation of globin gene structure and expression, and investigation of the phenomenon of hemoglobin switching whereby one hemoglobin is replaced by another during normal development. Our clinical projects are mainly directed toward devising or applying new therapeutic strategies. Many patients with chronic anemia require regular blood transfusions and often develop pathological iron overload. Efforts are directed toward defining the extent of organ dysfunction due to iron and to assessing the potential benefit of iron chelation in these patients. Various immuno-suppressive regimens are being compared as therapy for patients who have bone marrow failure which may be immunologically mediated.

Much of the work in the Clinical Hematology Branch relates to the general problem of the mechanism of regulation of the individual globin genes. In man and other species two gene clusters encode for the various globins which are produced in a highly ordered and sequential manner during ontogenic development. On chromosome 16 are found the genes for the embryonic α-like globin (ζ) closely linked to the genes for adult α globin, α_1 and α_2. The β-like genes, embryonic ε fetal γ, and adult δ and β are found on chromosome 11. The switch from Hb F ($\alpha_2\gamma_2$) to Hb A ($\alpha_2\beta_2$) during the perinatal period reflects selective expression of the closely linked and structually analagous γ and β globin genes. Our efforts have focused on elucidation of the manner in which this developmental switch in hemoglobin synthesis is regulated with the goal of attempting to devise some means to increase fetal hemoglobin synthesis in patients with severe disorders of hemoglobin structure or production.

The general structure of the globin genes and their linkage relationship has now been defined in man and various other species. Each gene contains two introns which divide the blocks of nucleotides which encode for the globin product. Various signals which are potentially important in regulating transcription of these genes have been identified just in front of the point at which RNA synthesis is thought to begin. Remaining to be devised is a suitable *in vitro* test system to systematically evaluate the function of these and other DNA sequences which may be important in modulating expression of the individual globin genes. We have constructed a cloning vector which may be useful or this purpose; it replicates both in bacteria and in monkey kidney cells. The vector includes the SV-40 origin of DNA replication while the monkey kidney cells constitutively produce SV-40 T antigen, a protein required for efficient DNA synthesis off the SV-40 replicon (individual project report: "Use of viral regulatory sequences to facilitate gene transfer and analysis of gene function"). The human δ and β globin genes have been subcloned into this vector and their expression has been studied. Accumulation of β-globin mRNA is 25-fold greater than that of δ globin mRNA. Thus the

relative level of expression of these genes in vivo is reproduced in our in vitro assay system. Nonetheless, our data also demonstrate that transcription initiation and termination of the β globin gene is probably random and certainly inaccurate although processing of β mRNA precursor-like species appears to be correct. We speculate that this inaccuracy in transcription is related to the way in which nucleosomes are positioned on the mini-chromosomes containing the δ or β globin genes and are devising experiments using a portion of the SV-40 genome to facilitate more "correct" positioning of nucleosomes with respect to transcriptional signals.

The thalassemias are congenital anemias characterized by deficient synthesis of one of the globin components of the hemoglobin molecule. In β thalassemia, a small amount of β globin may be produced or alternatively-β globin synthesis may be completely absent. Thus these disorders represent examples of regulatory mutations. Our analysis is focused on identifying the molecular lesions in patients with these disorders with the anticipation that insights into normal gene regulation may be acquired (individual project report: "Molecular defect in β thalassemia"). Data obtained in our laboratory and in others has established that errors or inefficiency in RNA splicing are frequent causes of β globin mRNA deficiency in the severe β thalassemias. Mutations (nucleotide substitutions) in the globin gene are copied into the globin mRNA precursors and are thought to create alternative splice sites or lead to inefficient removal of intervening sequence RNA. Globin mRNA precursors are present in exceedingly low concentration in erythroblast, e.g. roughly 100 molecules or less per cell - and thus highly sensitive probes are necessary for their detection and quantitation. We have devised two strategies for generating such probes and demonstrated abnormally spliced RNA species in the bone marrow cells of two patients. Currently we are collecting RNA and DNA samples from several additional patients with homozygous β thalassemia. These will be studied to detect and quantitate abnormal precursor molecules. Genes which appear to be of particular interest based on these data will be cloned and characterized by DNA sequencing and studied in the SV-40 based gene expression system described above.

The regions surrounding the globin genes are of interest because such regions may contain either regulatory loci or other structural genes. Identified to date downstream from the human β globin gene however, is only highly repetitive DNA. A repeated DNA sequence family, unusual because many of its members are extremely long - 6.4 kb - is represented by a copy just downstream from the β globin gene. Portions of this copy and another have been sequenced. The member adjacent to the human β globin gene exactly abuts a member of another moderately repetitive DNA sequence family, the so-called Alu repeat. The difficult question about moderately repetitive DNA is whether such sequences are functional or merely the nonessential and therefore uninteresting bi-products of DNA evolution.

An experimental animal which is highly suitable for investigation of hemoglobin switching is the sheep for this species exhibits a fetal to adult hemoglobin switch that is quite analagous to that which occurs in man. Furthermore, certain sheep have an additional β globin gene which is specifically activated during anemia, apparently by direct action of the hormone-erythropoietin-on erythroid cells. Thus the switch to Hb C production is a readily maniputable phenomenon which can be approached experimentally. The current goal of our molecular analysis of hemoglobin production in sheep is the characterization of the various globin genes (individual project report: "Regulation of the sheep globin genes").

Our desire is to deduce the general map of the β-like globin gene cluster, to determine the distances between the various genes, and to define their general structure. The fetal (γ) and adult (β^A) globin genes have already been cloned and characterized by restriction endonuclease mapping, heteroduplex analysis and DNA sequencing. During the past year we have identified an insertion-like element or transposon within the large intron of the γ globin gene. Because this element constitutes one of the major structural differences between the γ and β^A globin genes, it conceivably may be involved in modulating their differential expression although this element is moderately repetitive and occurs several thousand times elsewhere in the sheep genome. Our efforts to establish a linkage map of the sheep β-like globin genes has proceeded slowly because of the difficulty in isolating specific DNA fragments from genomic libraries using only globin gene sequence specific probes. Linkage between the β^C globin gene and an embryonci β-like globin gene has been established. A new cloning strategy which relies on a recombination probe should facilitate our efforts to walk the chromosome in either direction from the globin genes which have already been identified.

The goal of introducing functional genes into the cells of patients with monogenetic mutations and thereby providing genetic therapy for severe homozygous defects seems more realistic based on the recent explosion of knowledge about gene structure and the availability of several strategies to introduce gene into eukaryotic cells. Treatment of homozygous β thalassemia with periodic blood transfusions produces paliation but progressive iron accumulation with resulting organ dysfunction requires aggressive chelation therapy (individal project report: "Iron Chelation in Transfusional Hemochromatosis"). Removal of substantial amounts of iron with subcutaneously administered desferrioxamine may not prevent the development of cardiac complications in such patients. Similarly the currently available strategies for treatment of sickle cell anemia rely only on supportive care and periodic transfusion and thus are not satisfactory. These considerations provided us with a strong impetus to actively pursue the goal of providing gene therapy for these conditions.

Current strategies to introduce genes into cells _in vitro_ rely on the use of a selectable marker to insure proliferation of only those cells into which gene transfer has been effective. We are using the gene for the enzyme dihydrofolate reductase (DHFR). Increased concentrations of these enzymes render cells impervious to otherwise highly toxic concentrations of methotrexate. Hybrid viruses have been constructed in which the coding sequences for DHFR replace those for either the T antigen or coat proteins of SV-40. Such viruses cause an increase in DHFR activity during lytic infection of permissive monkey kidney cells although these viruses have not yet been shown to facilitate transfer of the DHFR marker gene into non-permissive cells. Alternative strategies which employ other resistance marker genes and adenovirus regulatory and replication signals are being explored. These studies are being pursued with a hope that useful genetic therapy may result but with the confidence that meaningful information about the regulation of gene expression will almost certainly be forthcoming.

Another aspect of erythropoiesis under active study in this Branch is the characterization of stem cells and the analysis of their differentiation. During the past several years we have utilized the various assays for erythropoietic progenitor cells to investigate the phenomenon of hemoglobin switching. Many interesting results were obtained although we have come to realize that the current assay systems are unlikely to produce additional new insights into globin gene

regulation. Hence we have been prompted to explore other avenues of investigation.

The general strategy which has been utilized is outlined in the individual project report: "Use of hybridoma technology in the study of erythroid differentiation". Hybridomas are clones of antibody producing cells derived by fusion of mouse myeloma cells with spleen cells from mice immunized with the antigen of interest. The power of this technology is that the antibodies produced, being the products of progeny cells derived from a single fusion event, have monoclonal specificity. Thus even when a mixture of antigens or a cell population having a diversity of surface determinants are used for immunization, after proper screening of many hybridoma clones, cells producing antibodies with unique specificity can be obtained.

The human K562 cell line was derived from a patient with chronic myelogeneous leukemia in blast crises. K562 cells behave like hematopoietic stem cells in that they have a large potential for self renewal and also exhibit phenotypic features characteristic of the erythroid, granulocytic, and megakaryocytic lineages. Therefore these cells were chosen as immunogens; we hope to derive hybridomas which produce antibodies which reacted with hematopoietic stem and progenitor cells but not with differentiated hematopoietic precursors. Antibodies with very diverse specificities have been obtained. Of greatest interest are those which appear to lyse progenitor cells by a complement mediated mechanism but which react little if at all with mature erythroid and myeloid precursors. These antibodies will be used to attempt to obtain enriched populations of hematopoietic progenitors using the fluorescent activated cell sorter. Other hybridoma antibodies have been shown to react with leukemic myeloblasts and may be useful for classifying the acute myelogeneous leukemias and also for detecting blast cells in the bone marrow and other tissues of patients in apparent hemotological remission.

Related to these immunological studies of hematopoietic differentiation are our efferts to define immunologically mediated bone marrow failure syndromes in man and to treat these conditions with immunosuppressive therapy (individual project report: "Hemopoiesis in bone marrow failure"). One regimen combines oral cytoxan and prednisone with either lymphocytopheresis or plasmapheresis in an effort to remove either suppressor T cells or antibody. Patients with pure red cell aplasia who exhibit a serum IgG inhibitor of erythroid colony formation have been shown to respond to this regimen. Six patients with severe acquired aplastic anemia have shown no response however, except for a mariginal improvement in one patient despite the presence of serum and/or cellular inhibitors of hematopoietic colony formation demonstrable in vitro. An alternative protocol which includes both high dose methylprednisone and anti-thymocyte globulin has been devised. The first patient with severe aplastic anemia treated with this regiman has shown a gratifying increment in total granulocyte count from the severely neutropenic to the normal range. Additional patients are being treated with this regimen.

The several projects within this Branch are related to the biosynthesis of hemoglobin. the regulation of production of specific hemoglobins, and the development and differentiation of erythroid stem cells. Strategies are being devised and tested which may facilitate gene transfer into hematopoietic progenitor cells and thereby make genetic therapy of the severe hemoglobinopathies feasible. From this comprehensive approach we hope to provide an experimental basis for optimal therapeutic strategies to treat the various red cell disorders.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02203 09 CHB

PERIOD COVERED October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)

Molecular Defects in β Thalassemia

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

	Name	Title	Affiliation
PI:	M. Pepe	Visiting Fellow	CHB NHLBI
	M. Goldsmith	Staff Fellow	CHB NHLBI
	T. Ley	Clinical Associate	CHB NHLBI
	J. Kantor	Guest Worker	CHB NHLBI
OTHER:	P. Turner	Medical Technologist	CHB NHLBI
	A.W. Nienhuis	Branch Chief	CHB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.0	0.5

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

These studies are directed toward determining the molecular lesion affecting β-globin production in patients with homozygous β-thalassemia. Earlier work in this laboratory and in others has established that ineffective removal of intervening sequence RNA from β globin mRNA precursors is a frequent cause of β globin mRNA deficiency in patients with severe β thalassemia. Our efforts during the past year have focused on attempts to define abnormal RNA precursors in bone marrow cells in patients with β thalassemia and to purify the globin genes from such patients by molecular cloning. Two types of abnormal RNA precursors have been demonstrated; one involves an abnormal splice within the first smaller intervening sequence and the second apparently involves a failure of ligation of an RNA molecule improperly spliced within the second larger intervening sequence. A new cloning strategy has been adapted in our laboratory which should facilitate rapid isolation of thalassemic globin genes.

PHS-6040
(Rev. 2-81)

Objectives:

The objective of these studies is to use the naturally occuring model of defective gene expression provided by β-thalassemia to attempt to understand gene regulation. Patients with this disorder make less than adequate quantities of a normal polypeptide and thus may be said to have regulatory mutations. Recognition of certain molecular lesions in this disease may provide clues as to the specific mechanism for regulation of expression of individual genes in human cells. Furthermore, appreciation of the molecular basis of this disorder may suggest an approach to specific therapy.

Methods:

1. Characterization of globin mRNA precursors: Nuclear or total poly-adenylated RNA from human bone marrow cells is fractionated by electrophoresis in agarose gels containing methyl mercury. The RNA is transferred to diazotized cellulose paper and annealed to probes specific for either intron or coding sequences. The large intron from the normal human β globin gene has been subcloned into the plasmid, pBR322, and purified in large quantities. After 5' or 3' end labeling, the two DNA strands are separated by gel electrophoresis and annealed to bone marrow RNA. Following S_1 digestion, mRNA precursor molecules are identified by size fractionation on acrylamide gels following denaturation according to the Berk and Sharp technique.

2. Preparation of single stranded globin gene probes by cloning into the phage M13: The bacteriophage, M13, secretes one of its two strands into the culture media. Various portions of the normal human β globin gene have been subcloned into this vector and secreted single strands used as a template to synthesize uniformly and highly labeled single stranded probes. These are used to characterize globin mRNA precursors by the Berk and Sharp technique as described above.

3. Cloning of thalassemic globin genes: Seed and Maniatis have developed a technique to screen genomic DNA bacteriophage libraries by use of a "recombination probe" subcloned into a plasmid vector. The bacteriophage library is amplified in a bacterial strain which harbors the vector, π vx, into which has been inserted a small segment of the gene of interest or its flanking region. During amplification, recombination occurs so that the π vx plasmid is inserted into these cloned DNA segments in the Charon 4A library which contain sequences homologous to its "probe" fragment. Because π vx also contains the gene for suppressor F tRNA, Charon 4A clones which acquired this plasmid by homologous recombination are subsequently able to form phage plaques on a bacterial strain which lacks the suppressor tRNA. This occurs because Charon 4A has amber mutations in genes for critical coat proteins. This strategy is currently in use in our laboratory and should greatly facilitate the molecular cloning of thalassemic genes.

Major Findings:

1. Characterization of globin mRNA precursors: Using single strand probes specific for RNA sequences transcribed from the large intron of the β globin gene, we have obtained additional evidence that there is an abnormal but relatively stable precursor RNA species in the cells of one black patient. This species

contains RNA transcribed from the 3' half only of the large intron suggesting that it has been cut but not spliced properly during processing. A single stranded probe obtained using the M13 cloning system has been used to demonstrate the presence of an RNA molecule abnormally spliced within the small intron and present within the cells of an Italian patient with homozygous β thalassemia.

2. Molecular cloning of β thalassemia globin genes: The plaque hybridization technique for identifying bacteriophage containing cloned globin genes has not been successful in attempting to identify such clones in libraries of thalassemic DNA. New genomic DNA fragment libraries have been prepared. The π vx screening system has been shown to work in our hands in control experiments and currently we are screening several libraries using this strategy.

Significance to Biomedical Research in the Institute Program:

Homozygous β-thalassemia is a disease which causes severe morbidity and mortality to its victims. Thus an understanding of the genetic basis of this disorder may provide a basis for designing therapy which could be of extraordinary benefit to these individuals. Furthermore, this disease is prototype of a human regulatory genetic mutation and thus may provide insight into the mechanism of gene regulation in human cells.

Proposed Course of the Project:

Our immediate goals include definition of the normal processing intermediates for the human globin mRNA precursors by use of techniques which allow definition of specific splice sites used in the removal of intervening sequence RNA. We hope to study RNA from the cells of a number of patients with homozygous β^+-thalassemia thereby expanding our knowledge of the processing defects occurring in this condition. These analysis may provide insight into the processing pathway and indicate whether certain splicing sites are obligatory for production of normal amounts of mature β globin mRNA. We intend to continue our efforts to obtain pure globin genes affected by β^+-thalassemia mutation by using the molecular cloning recombinant DNA techniques. Sequencing of specific splice sites may allow definition of the exact mutations which result in abnormal processing. Insertion of large introns from the β globin gene into SV-40 vectors may facilitate definition of processing intermediates once the cloned thalassemic genes are obtained.

Publications:

1. Kantor, J.A., Turner, P.H., and Nienhuis, A.W. Beta^+ thalassemia: mutations which affect processing of the β globin mRNA precursor. Cell, 21:145-157, 1980.

2. Tam, J.O., Kaufman, R.E., and Nienhuis, A.W. Analysis of globin gene structure in patients with β thalassemia by restriction endonuclease mapping. Hemoglobin 5(3), 209-215, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 H1 02204 09 CHB

PERIOD COVERED October 1, 1981 - September 30, 1982

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

PI:	A.W. Nienhuis	Chief, Clinical Hematology Branch	CHB NHLBI
	J.E. Barker	Cell Biologist, Jackson Laboratories	
OTHER :	J.E. Pierce	Chief, Laboratory Animal Med. & Surg.	NHLBI
	L. Stuart	Chief, Ungulate Section, NIH Animal Center	
	D. Buckhold	Veterinary Officer	NHLBI

COOPERATING UNITS (if any)
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Ungulate Section, NIH Animal Center
Jackson Laboratories, Bar Harbor, Maine

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 0.3	PROFESSIONAL: 0.3	OTHER: 0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The purpose of these studies is to establish the basic developmental and cellular events operating in regulating the genes for fetal (γ) and adult (β^A and β^C) globin in sheep erythroid cells. Induction of Hb C may be achieved in vitro or in vivo by exposure of erythroid stem cells to high erythropoietin (epo) concentration. The amount of Hb C synthesis appears to be related to the degree of differentiation of stem cells which give rise to erythroid colonies in vitro; the earliest stem cells give rise to colonies making more Hb C. The perinatal switch from Hb F ($\alpha_2\gamma_2$) to Hb A ($\alpha_2\beta^A_2$) also appear to be regulated in hematopoietic stem cells although the progenitors which can be assayed in vitro appear to have already been committed with respect to the hemoglobin produced in the erythroblasts which are their progeny. We have developed a transplantation model to investigate whether such committment is intrinsic to the stem cells or related to the micro-environment of these cells. Successful engrafment of stem cells from an animal at the immediate pre-switch stage into a mid-gestation fetus has been documented in one fetus although no adult hemoglobin production was found.

PHS-6040
(Rev. 2-81)

Objectives:

The primary objective of this project is to determine the normal mechanism of regulation of expression of globin genes during ontogeny in sheep. Specifically, we wish to learn the relative importance of stem cell committment vs hematopoietic micro-environmental influence in regulating the peri-natal switch from fetal to adult hemoglobin formation.

The globin genes of sheep serve as a suitable model for investigating these phenomenon since the several individual globin genes are expressed only during specific developmental periods. Thus the γ globin gene (Hb F = $\alpha_2\gamma_2$) is expressed during fetal life while the allelic adult β globin genes ($\beta^A\beta^B$) are expressed in adult animals making 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 = $\alpha_2\beta_2^C$) is expressed transiently in new born animals and in animals made anemic. Induction of β^C globin synthesis is specifically stimulated by erythropoietin (epo) both in vivo and in vitro. This readily maniputable switch in hemoglobin production, namely the epo induced synthesis of the Hb C has proved to be a useful tool for our studies.

Methods:

Fetuses of accurately defined gestational age are selected for transplantation experiments. Donor animals which are homozygous for the β^A globin gene range in age from 125 to 135 days. Such fetuses are surgically removed from the uterus and the bone marrow cells recovered by suspension in sterile tissue culture media. Recipient 70-80 day fetuses which are homozygous for the β^B globin gene, are given infusions of these bone marrow cells. The recipient fetuses are exteriorized from the uterus surgically, the bone marrow cells infused via the umbilical vein, and the fetuses are replaced in the uterus. Gestation is allowed to proceed for 10-20 days. At that time, the recipient fetuses are sacrificed and their bone marrow cells are recovered. Incubation of these cells in suspension culture in the presence of [^{3}H]leucine is performed to label the globin chain. Cells are also placed in plasma clot culture. After erythroid colony formation is complete (5-7 days) [^{3}H]leucine is added for 24 hours. The radio-labeled globins are resolved by ion exchange chromatography.

Major Findings:

Three recipient fetuses have survived the surgical manipulations employed in the transplantation experiments although the analysis is complete on samples from only one of these animals. Successful engrafment was documented because the erythroid colonies which formed in vitro made Hb C ($\alpha_2\beta_2^C$). The recipient cells lacked the capacity to form this hemoglobin since this animal was homozygous for the β^B allele; such animals do not have the gene for β^C globin. No evidence for adult hemoglobin synthesis ($\alpha_2\beta_\beta^A$) was noted in vivo in the recipient. These initial results suggest that stem cells which would normally complete the perinatal switch in their program of committment from fetal to adult hemoglobin formation, failed to do so when transplanted into an earlier gestational stage.

Significance to Biomedical Research and the Program in the Institute:

Several human anemias (e.g., β-thalassemia and sickle cell anemia) are char-

acterized by abnormal globin synthesis or function. A common feature of these anemias is that synthesis of fetal hemoglobins is normal during fetal life. Human hereditary persistence of fetal hemoglobin is a mild anemia and requires minimal clinical management. A rational approach to treatment of anemia involving defects in globin synthesis is, therefore, the therapeutic re-utilization of the fetal gene in adult life. Attempts to describe the basic cellular and hormonal regulation of erythropoiesis in sheep are done with the goal of realizing this possibility.

Proposed Course of Experiments:

Our previous data appear to suggest that the stem cells from either fetuses or adult animals are committed with regard to their program of hemoglobin synthesis. Therefore, the most germane question is whether committment is intrinsic to the stem cells or related to the micro-environment. The transplantation experiments are designed to provide data pertinent to this question. Dr. Jane Barker who has been the principal investigator on this project for several years, has left to work at the Jackson Laboratory. Her involvement in this project will terminate when the transplantation experiments already initiated are complete. Therefore, a reassessment of our approach to the problem of hemoglobin switching has been necessary. The most readily achievable goal appears to be that of characterizing the non-α globin gene region completely by use of the molecular cloning techniques as outlined in the project "Regulation of the Sheep Globin Genes". Identification of specific proteins which interact to modulate globin gene expression in erythroid cells would then be a reasonable goal and maybe the most fruitful way to investigate the hemoglobin switching mechanism.

Publications:

1. Barker, J.E.: Hemoglobin switching in sheep: Characteristics of BFU-E derived colonies from fetal liver. Blood 56:495-500, 1980.

2. Barker, J.E., Pierce, J.E., Nienhuis, A.W.: Hemoglobin switching in sheep: A comparison of the erythropoietin induced switch from Hb F to Hb C and the fetal to adult hemoglobin switch. Blood 56:488-494, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02206 08 CHB

PERIOD COVERED

October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)

Regulation of Sheep Globin Genes

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

PI:	Helen Coon	Biologist	CHB NHLBI
	M. Goldsmith	Staff Fellow	CHB NHLBI
Other:	A. Davis	Chemist	CHB NHLBI
	M. Harrison	Biologist	CHB NHLBI
	A.W. Nienhuis	Branch Chief	CHB NHLBI

COOPERATING UNITS (if any)

Section on Laboratory Medicine and Surgery, NHLBI; Ungulate Section, NIH Animal Center

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The purpose of this project is to define the mechanism or regulation of the individual globin genes during erythroid differentiation. Current efforts are focused on isolating the several globin genes from a library of sheep genomic DNA fragments which had been cloned into E. coli using the bacteriophage vector-Charon 4A. Previous studies had identified the sheep γ and β^A globin genes and had shown that these genes lie within 8 kb regions of general sequence homology. An additional clone ($\lambda SG\beta^C 52$) has been isolated from our Charon 4A library. This clone is of particular interest because at its 5' end are DNA sequences which represent the 3' end of the β^C globin gene. On the other end of this cloned DNA fragment is a globin gene which has been shown previously to encode for an embryonic globin. Thus the linkage between the β^C globin gene and an embryonic globin gene has been established. Both the large introns of the β^A and γ globin genes have been shown to contain repetitive DNA. Complete DNA sequencing of the γ globin gene large intron has revealed an element bounded by short tracts of both direct and inverted repeated DNA sequences. These features are characteristic of insertion-like or transposable elements.

PHS-6040
(Rev. 2-81)

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.

Techniques are now available which make it possible to completely define gene structure and to determine the homologous regions surrounding the various globin genes. This experimentation involves the use of recombinant DNA technology and characterization of the isolated recombinants by restriction endonuclease mapping, nucleotide sequencing, and heteroduplex mapping by electron microscopy. Our current goal is to define completely the beta globin region in sheep at the DNA sequence level.

Methods:

1. Identification of bacteriophage recombinants containing globin genes and/or surrounding sequences: Two general procedures have been employed during the past 12 months. The first involves plaque hybridization using a radioactive probe specific for globin gene sequences or for sequences previously shown to be uniquely represented in the sheep globin gene region. A technically far simpler strategy has recently been developed. This technique involves the use of a bacterial strain which harbors a plasmid which contains both a gene for the suppressor F tRNA and also the DNA sequence for which one is probing the genomic DNA fragment library. Amplification of the bacteriophage library in this host leads to homologous recombination between the vector and any bacteriophage containing its homologous sequences. Subsequent plating of the amplified library on a host which lacks the suppressor tRNA gene allows formation of phage plaques only if homologous recombination has occurred.

2. DNA sequence analysis: This high resolution but somewhat laborous technique can be applied to specific fragments of interest; most commonly these are restriction endonuclease fragments which contain globin gene sequences. The goal of this type of analysis is to identify the globin gene represented in a particular recombinant. After a restriction endonuclease map of an individual recombinant is obtained, specific DNA fragments are isolated. Individual fragments are labelled on the ends by the polynucleotide kinase (5') or DNA polymerase I (3') reaction. The labelled fragment is cut with another restriction endonuclease and the two [^{32}P] ends are separated by polyacrylamide gel electrophoresis. The DNA nucleotide sequence can then be obtained by partial chemical degradation of the individual end-labelled fragments followed by polyacrylamide gel electrophoresis and radioautography.

3. Analysis of regions surrounding globin gene sequences: To compare the sequence homology surrounding two individual globin genes, DNA derived from recombinants containing these globin genes can be annealed and the resulting heteroduplexes examined by electron microscopy. Homologous regions form duplexes whereas the non-homologous regions remain single stranded; these can be distinguished in the resulting electron micrographs.

4. Determination of repetitive frequency of DNA sequences contained within specific recombinants: Moderately repetitive DNA sequences interspersed among structural gene can be demonstrated by one of two techniques. Total genomic DNA from sheep is rendered radioactive by a nick-translation reaction and annealed to DNA fragments obtained by restriction endonuclease digestion of DNA from individual clones followed by agarose gel electrophoresis. The intensity of hybridization in a Southern blot analysis, to specific fragments is a measure of the repetitive frequency of sequences within that fragment. Alternatively, the clone may be rendered radioactive, digested with specific restriction endonucleases, the fragments resolved by gel electrophoresis, and the gel blotted against total genomic DNA immobilized on a filter. Again the intensity of the radioautographic signal from specific bands is a measure of the repetitive frequency of that DNA sequence.

Major Findings:

1. Characterization of the γ globin gene of sheep: Previously we had identified four overlapping cloned DNA fragments of total length of 28 kb all of which contained part or all of the γ globin gene. An additional clone has been identified (λSGγ32) which extends this region another 3.0 kb upstream. As of yet no other globin gene has been identified linked to the γ globin gene.

2. Linkage of the sheep β^C globin gene to an ε-like globin gene: A recombinant bacteriophage which contains the 3' end of the β^C globin gene also contains an entire ε-like globin gene some 14 kb downstream. The β^C globin gene was identified by DNA sequencing. Using the mini-plasmid screening system described by Seed and Maniatis, 70 or more recombinant bacteriophage containing the 1.2 kb Hind III/Pst I fragment downstream from the ε-like globin gene have been isolated and are available for the study.

3. Repetitive DNA sequences in the sheep globin gene introns: Both the fetal γ-globin and adult β^A globin large introns contain repetitive DNA sequences. There are approximately 15,000 copies of the sequence found in the γ globin gene intron and about 25 fold fewer copies of the sequence found in the β^A globin gene. The entire γ globin gene large intron has been sequenced and an insertion-like element has been identified. The sequence is 280 base pairs long and is bounded on each end by inverted repeated DNA sequences 6 bp long. In addition, adjacent to this repetitive element on either end is a direct repeat sequence of 13 bp. Identification of this sequence in the sheep γ globin gene intron was facilitated by comparison of the DNA sequence data to that of the goat γ and β^A globin genes obtained by J. Lingrel and his collaborators.

4. γ globin mRNA precursors: Electrophoretic analysis of fetal liver polyadenylated RNA has identified several γ globin RNA precursor molecules longer than mature mRNA. The lengths of approximately 1600, 1200, and 1000 nucleotides suggest that there are probably at least 2 principle splicing sites in the large intron.

Proposed Course of the Project:

Screening of the sheep genomic DNA fragment libraries will be continued in order to obtain additional clones containing the β^C globin gene and also in an effort to construct a map of the entire globin gene region. The γ and β^A globin genes will be sequenced in their entirety thereby facilitating further definition of the moderately repetitive DNA sequences found within their large introns. Heteroduplex analysis will be pursued in an effort to obtain clues regarding non-homologous regions which may have regulatory function.

Publications:

1. Kretschmer, P.J., Coon, H.C., Davis, A., Harrison, M., and Nienhuis, A.W. Hemoglobin switching in sheep: Isolation of the fetal γ globin gene and demonstration that the fetal γ and adult β^A globin genes lie within eight kilobase segments of homologous DNA. J. Biol. Chem. 256:1975-1982, 1981.

2. Kretschmer, P., Coon, H.C., Davis, A., Harrison, M. and Nienhuis, A.W. The structure and organization of the sheep globin genes. The Second Conference on Hemoglobin Switching. In Stamatoyannopoulos, G., Nienhuis, A.W. (Eds.) Organization and Expression of Globin Genes. New York, Alan R. Liss, Inc. 1981. Vol. I pp 59-68.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02208 07 CHB

PERIOD COVERED
October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)

Iron Chelation in Transfusional Hemosiderosis

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

PI:	A.W. Nienhuis	Branch Chief	CHB NHLBI
	Patricia Griffith	Clinical Nurse Specialist	CC
Other:	T. Ley	Clinical Associate	CHB NHLBI
	W.F. Anderson	Laboratory Chief	LMH NHLBI
	M. Gaul	Clinical Nurse Specialist Montreal Children's Hospital Montreal, Quebec, Canada	
	H. Strawczynski	Director, Chronic Care Clinic, Montreal Children's Hospital Montreal, Quebec, Canada	

COOPERATING UNITS (if any)

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Thalassemic Clinic, Montreal Children's Hospital, Montreal, Quebec, Canada

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 2.0	PROFESSIONAL: 2.0	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)

These studies are designed to evaluate the clinical benefits achieved by iron chelation in patients with chronic iron overload. Desferrioxamine is administered by subcutaneous infusion and iron removal is determined by quantitation of urinary iron excretion and careful recording of the total iron administration by transfusion. Those patients which have no evidence of cardiac disease are randomized to receive either ascorbic acid (3 mg/kg) or placebo. Sixty-five patients are now included in our long term chelation trial and of these 49 have been randomized to the ascorbic acid study. Most patients have now been followed for a minimum of two and many for three years. Currently all patients are undergoing annual evaluation with the expectation that we will analyze all data in an effort to judge the clinical efficacy of chelation and the influence of ascorbic acid therapy.

PHS-6040
(Rev. 2-81)

Objectives:

The objectives of these studies are to evaluate available iron chelators to maximize their effectiveness, and to test new chelators as they become available. An effort is being made to develop clinical criteria which will be helpful to determine the efficacy of chronic chelation therapy. A randomized trial of supplemental ascorbic acid has been initiated to assess the value and/or toxicity of this agent in promoting mobilization of iron by desferrioxamine in patients with iron overload.

Methods:

Patient populations which participate in these studies include: 1) patients with transfusion dependent congenital or acquired anemia who require regular blood transfusions to sustain life, and 2) patients with idiopathic hemochromatosis at various stages in the process of iron removal by phlebotomy.

Clinical evaluation of organ function include the following:

1) Heart: An estimate of cardiac size by chest X-ray and electrocardiographic analysis is obtained. Echocardiographic studies are obtained to determine anatomical dimensions of the left ventricle and left ventricular function as assessed by resting ejection fraction.

2) Endocrine evaluation includes specific testing of the pituitary, thyroid, adrenal, pancreatic islets, and gonad function by baseline measurements and various provocative tests.

3) Liver function is determined by standard clinical testing. In addition, liver biopsies are performed to assess histology and to quantitate liver iron concentration.

4) Serial serum ferritin measurements are obtained to assess the utility of this parameter in estimating total body iron stores and also to follow the course of iron removal.

Major Findings:

No major new observations have proceeded from our study during the past 12 month follow-up period. No significant toxicity of the Desferal-ascorbic acid regimen is clinically obvious. Analysis of all the data collected over the past four years is planned during the next several months.

Significance to Biomedical Research and to Institute Program:

Hemosiderosis is a major cause of morbidity and mortality in patients requiring prolonged transfusion therapy. The role of iron chelators in improving the clinical course of these patients must be ascertained.

Proposed Course of the Project:

This project will be continued until a suitable iron chelator is found and evaluated or until the need for transfusion therapy in thalassemia and other congenital hemolytic anemias is removed. The majority of our series of thalassemic patients on the chelation trial have had two or more annual evaluations. Within the next several months we anticipate analysing the follow-up studies and comparing the results to baseline data with particular attention to cardiac function. From this analysis we hope to learn whether there is any difference in the clinical course of the patients on ascorbic acid compared to those on placebo and therefore to determine whether this phase of the trial need be continued.

Publications:

1. Nienhuis, A.W., and Propper, R.D.: The thalassemias: disorders of hemoglobin synthesis. In Hematology of Infancy and Childhood. (D.G. Nathan and F. Oski, eds) Philadelphia, Saunders, Vol. 1:726-800, 1981, Second Edition.

2. Nienhuis, A.W. and Griffith, P.: Thalassemia. In Gellis, S.S. and Kagan, B.M. (Eds.): Current Pediatric Therapy, Tenth Edition. W. B. Saunders, Phila. 1981. In Press.

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4. Nienhuis, A.W. Vitamin C and Iron. New Eng. J. of Med. 304:170-171. January, 1981.

5. Cannon, R.O., III, Dusheiko, G.M., Long, J.A., Ishak, K.G., Kapur, S., Anderson, K.D. and Nienhuis, A.W. Hepatocellular Adenoma in a Young Woman with β-Thalassemia and Secondary Iron Overload. Gastroenterology. In Press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02304 04 CHB

PERIOD COVERED
October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)

Hematopoiesis in Bone Marrow Failure

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

PI:	Neal S. Young	Expert	CHB	NHLBI
Other:	B. Hurson	Biologist	CHB	NHLBI
	Keith Humphries	Visiting Associate	CHB	NHLBI
	Warren Ferris	Clinical Associate	CHB	NHLBI
	Harvey Klein	Assistant Chief	BB Dept.	NIH
	Arthur W. Nienhuis	Branch Chief	CHB	NHLBI

COOPERATING UNITS (if any)

Blood Bank, CC, NIH

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3-1/4	3	1/4

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Multiple cellular interactions are required for normal hematopoietic cell differentiation. Abnormalities in these interactions may result in bone marrow failure, especially aplastic anemia. Evidence has accumulated that a suppressor T lymphocyte may be the etiologic agent in some cases of bone marrow aplasia. We have completed a study of the effects of plasmapheresis and lymphocyte depletion in combination with cyclophosphamide and prednisone on patients with pure red cell aplasia and aplastic anemia. A study of the effects of anti-thymocyte globulin in chronic and acute cases of aplastic anemia is in progress. Treated patients and those with partially remitted disease are being studied by using in vitro methods that examine the effects of lymphocyte subpopulations on hematopoietic colony growth and enumerate the number of helper and suppressor T lymphocytes.

PHS-6040
(Rev. 2-81)

Objectives:

The maturation and proliferation of a hematopoietic stem cell to its final products - platelets, granulocytes, and erythrocytes - is dependent upon humoral growth factors and cellular interactions. The precise nature of the interactions among stem cells, lymphocytes, and monocytes is unknown. For example, in some studies removal of T lymphocytes abrogates erythroid colony growth. In other studies, monocytes appear to play a more primary role and may be acted upon by T lymphocytes. Similarly, the role of lymphocytes and monocytes in aplastic anemia, a disease characterized by the physical absence of bone marrow cells and decreased blood cell production, is confusing. Multiple lines of evidence suggested an immune etiology for at least some cases of aplastic anemia. Immunosuppression which precedes bone marrow transplantation has resulted in recovery of autologous bone marrow function in some patients. An occasional syngeneic twin has been unable to accept his bone marrow transplant in the absence of immunosuppressive therapy. In vitro studies also have suggested a role for a suppressor cell on hematopoiesis. Peripheral blood and bone marrow cells of some patients with aplastic anemia inhibit normal hematopoiesis as assayed by in vitro colony formation. Humoral factors that inhibit hematopoiesis have also been identified in some patients. Various studies have localized suppressor effects to T lymphocytes, a subpopulation of T lymphocytes which have immunoglobulin on their cell surfaces, B cells, and monocytes. Finally, European trials have shown a favorable effect of anti-thymocyte globulin, derived from the sera of horses immunized with human thymocytes, on the course of patients with aplastic anemia; in some studies, improvement rates have exceeded those obtained by bone marrow transplantation.

We have performed studies on aplastic patients which attempt to correlate clinical course, response to therapy, and experimental laboratory findings. In an initial study, patients with aplastic anemia who fulfilled the criteria for severe disease have been treated with immunosuppressive therapy, consisting of intensive plasmapheresis or lymphocyte depletion in combination with cyclophosphamide and prednisone. The number of erythroid and myeloid hematopoietic colonies, the presence and titer of humoral inhibitors of hematopoiesis, and inhibition by peripheral blood or bone marrow cells of normal hematopoiesis in culture have been correlated with response to this treatment. In a second study now in progress, both patients from the first study as well as new referrals are being treated with anti-thymocyte globulin in combination with high doses of methylprednisolone and androgens. In addition to measurement of hematopoietic colony formation, the number of helper and suppressor T lymphocytes, monocytes, and Ia bearing lymphocytes in the peripheral blood are being measured using the fluorescent activated cell sorter. A population of patients with stable, partially remitted aplastic anemia are being studied in a parallel fashion. Finally, the effects of monoclonal antibodies specific for different cell types, including hybridoma antibodies prepared against K562 cells in our laboratory (see Annual Report # Z01 HL 02309 02 CHB) are being examined for their effects on normal and abnormal hematopoiesis.

Methods:

All treated patients with aplastic anemia fulfill the criteria for severe disease, as defined by peripheral blood counts in the presence of a hypoplastic bone marrow biopsy. In the first clinical study, patients underwent plasmapheresis or lymphocyte depletion 3x weekly for 1 month in combination with cyclophosphamide

2 mg/kg/day, and prednisone 1 mg/kg/day for 1 month. In the treatment of patients with anti-thymocyte globulin, Upjohn ATG has been administered at a dose of 15 mg/kg/day daily for 14 days and then on alternate days for a further 14 days. Methylprednisolone is administered concurrently beginning at doses of 20 mg/kg/day intravenously with gradual tapering over 1 month. Oxymetholone is given at a dose of 2 mg/kg/day for 2 months. Blood counts, serum chemistries, quantitative immunoglobulins, complement, and responsiveness to skin tests has been assayed periodically in treated patients.

Hematopoietic colonies are grown in methylcellulose in the presence of either placental condition media to stimulate myeloid colony development or phytohemagglutinin stimulated leucocyte condition media and erythropoietin to stimulate erythroid colony growth. Our conditions are such that removal of the adherent cells, which normally produce either colony stimulating or burst promoting activity, affects neither the size nor number of colonies formed under these growth conditions. To assay serum inhibitors of hematopoiesis, bone marrow or peripheral blood cells from normal donors are incubated with patient serum, in the presence of autologous human serum as a source of complement, for 2 hours at 37°C; the cells are then washed free of excess antibody and complement and plated in semi-solid media as described above. To determine cellular inhibition, the bone marrow or peripheral blood cells from normal donors and patients with aplastic anemia are incubated at 37°C for 2 hours prior to co-culturing. Colonies derived from the myeloid precursor CFU-C are counted at 10 days and 21 days. CFU-E are counted at 5-7 days and must consist of at least 8 hemoglobin-containing cells in a tight cluster. Colonies derived from the most primitive erythroid progenitor, the BFU-E, are counted at 12 and 18 days.

Inhibition studies of normal and abnormal hematopoiesis have employed monoclonal antibodies commercially prepared by Orthro and Becton-Dickinson, at concentrations determined to be optimal from fluorescent cell sorting experiments. Monoclonal antibodies which have activity against K562 leukemia cells are derived from hybridoma supernatants or ascites fluid prepared in our laboratory. Bone marrow and peripheral blood cells are incubated for 2 hours at 37°C with the monoclonal antibodies and rabbit serum as a source of complement and washed prior to their plating in methylcellulose.

Fluorescent activated cell sorting has been performed using the Becton-Dickinson FACS II and the monoclonal antibodies described above. Adherent cells have been removed in some experiments to diminish the background of non-specific binding due to monocyte-macrophage F_c receptors. In other experiments, unfractionated preparations have been used and the binding to these cells subtracted.

Results:

A. Clinical Trials

Three patients with pure red cell aplasia and six patients with aplastic anemia have been treated with plasmapheresis or lymphocyte depletion and immunosuppression. All three patients with pure red cell aplasia demonstrated a response, ranging from complete but temporary remission to a 25% decrease in red cell transfusion requirement or remission as measured by reticulocytosis and the appearance of normoblasts in the bone marrow. In contrast, only 1 of 6 patients with aplastic

anemia showed a response to therapy. This patient had had an atypical, fluctuating course with sporadic reticulocytoses and the persistence of hematopoietic cellular elements in the bone marrow.

One of the patients who failed on the regimen of plasmapheresis and immunosuppression has been treated with anti-thymocyte globulin, high doses of methylprednisolone, and oxymetholone. Following a one month course of treatment, the patient demonstrated a marked leukocytosis, with a rise in the absolute granulocyte count from an average of $400/mm^2$ pre-treatment to 1400 following therapy. A mild reticulocytosis and improvement in platelet transfusion requirements followed the increase in white blood count.

B. Laboratory

All the patients with aplastic anemia have shown a marked decrease in erythroid and myeloid progenitors as measured by colony assays. Patients with pure red cell aplasia show a selective decrease in erythroid precursors, more striking and consistent for the primitive BFU-E compared to the more mature CFU-E. No improvement has been measured in colony number for either the aplastic patient who responded to lymphocyte depletion or either of two patients with pure red cell aplasia who responded to plasmapheresis. However, a qualitative improvement in cellular differentiation was noted in colony studies in these cases.

In the patient with aplastic anemia who responded to lymphocyte depletion, evidence of co-culture inhibition was present before therapy and absent at its completion, consistent with his clinical improvement with immunosuppression. However, in other aplastic patients in whom humoral suppressors were noted, treatment either had no effect or was not correlated with decreases in their activity. In contrast, in two patients with pure red cell aplasia who responded to treatment, there was a decrease in humoral suppressor activity. However, no attempt was made to treat patients with either lymphocyte depletion or plasmapheresis based on the laboratory determination of the presence of a cellular as compared to humoral suppressor.

Studies of suppressor and helper lymphocyte subpopulations in patients with aplastic anemia have so far shown marked increases in peripheral blood suppressor lymphocytes in both patients with severe depression of blood counts undergoing treatment and patients with partially remitted disease whose blood counts have improved so that they no longer receive transfusions. A similar increase in suppressor cells is present in the bone marrow, but contamination with peripheral blood bone marrow samples prior to FACS analysis has not been excluded. In the patient who has responded to anti-thymocyte globulin, there has been a decrease from 70% to 20% in the peripheral blood suppressor proportion of lymphocytes.

Proposed Course of the Project:

A total of 10-12 patients with aplastic anemia will be treated with anti-thymocyte globulin. An effort will be made to treat all the patients who failed to respond to medical immunotherapy as well as referred patients with either stable or acute courses.

Changes in lymphocyte subpopulations may be measured accurately from peripheral blood. However, the problem of contamination of bone marrow specimens by

peripheral blood remains an unsolved one. Efforts are being made to devise alternative methods of performing microscopic fluorescence on bone marrow aspirates and bone marrow biopsies so that these cells may be qualitatively assessed. The group of patients with partially remitted aplastic anemia may be a valuable research source, as they are generally free of metabolic stress such as infection. In addition, they have an adequate number of cells present for appropriate studies. These patients may be ideal subjects for short-term experiments which measure the effects of drugs on hematopoiesis and specific cell populations.

In vitro experiments using monoclonal antibodies may be a simple, highly accurate method of separating helper and suppressor effects of lymphocytes and monocytes and defining the exact cellular relationships in hematopoiesis.

Publications:

1. Young, N.: Aplastic anemia: Research themes, clinical issues. In "Progress in Hematology, Vol. XIII". E. Brown (ed.), 1981, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02306 03 CHB

PERIOD COVERED
October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)
Transformation of Mammalian Cells

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

PI: M.J. Chen	Senior Staff Fellow	CHB, NHLBI
OTHER: A.W. Nienhuis	Branch Chief	CHB, NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH Bethesda, Maryland 20205

TOTAL MANYEARS: 0.75	PROFESSIONAL: 0.75	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 transformation of cultured mammalian cells with defined DNA fragments has been explored as a system for studying eukaryotic gene regulation with a special interest in studying the expression of normal human globin genes. The co-transformation of mouse fibroblasts with herpes simplex thymidine kinase gene and a recombinant bacteriophage, λHβG1, containing both the human δ and β globin genes has resulted in mouse cell lines in which the human globin genes are stably integrated without apparent rearrangment. The human globin genes in transformed mouse fibroblasts were found to be virtually unmethylated. Although undermethylation of DNA seems to correlate with gene activity in normal cells, the level of expression of human globin genes in the co-transformed mouse fibroblasts was much less than that found in human erythroblasts. Nor does undermethylation insure correct transcription since the 75-100 copies of human β globin mRNA found in each cell were seemingly somewhat shorter than the authentic mRNA species.

Objectives:

Due to the development of DNA splitting and gene cloning techniques, certain specific genes other than those of viral origin can now be obtained in a homogenous state and in large quantity. Consequently, it is possible to study the organization of such genes by restriction endonuclease mapping and to depict the primary sequence by rapid DNA sequencing techniques. Such study constitutes the first step toward the ultimate goal of understanding the fine regulation of gene expression which in turn is essential for understanding differentiation, development, and cancer.

Herpes simplex viral thymidine kinase gene (Herpes TK gene) has been found to transform thymidine kinase negative mouse fibroblast cells in culture into TK positive transformants, selectable in HAT medium. Co-transformation with a second non-selectable gene is also a frequent occurrence if a DNA fragment including that gene is used along with Herpes TK gene in the transformation experiment. Our studies are directed toward exploring the usefulness of the co-transformation system for studying globin gene regulation. Cloned genomic globin genes are introduced into recipient cells and their expression is studied by examining extracts of the cells for RNA transcripts and globin

Methods:

1. Propagation of transformed mouse fibroblasts and preparation of DNA: Cells previously transformed with a plasmid vector containing the Herpes TK gene along with λHβG1 (a bacteriophage containing the human δ and β globin genes) were grown in selective medium. High molecular weight DNA was prepared from each transformed cell clone using Blin's procedure.

2. Characterization of globin mRNA transcripts: RNA was prepared from the transformed cell lines by standard techniques. β globin mRNA sequences were quantitated in total cellular RNA by hybridization in solution to a single stranded pure β globin specific DNA probe. Sizing of the globin gene transcripts was by Northern blot analysis.

3. Analysis of the methylation status of certain cytosine residues is possible through use of restriction endonucleases which are sensitive to methylation. Among the most useful are the pair of isoschizomers, Hpa II and Msp I. These enzymes share the recognition sequence, CCGG, but Hpa II is sensitive to methylation of the internal C residue while Msp I is not. Msp I is sensitive to methylation of the outer C residues so lack of digestion with both enzymes provides useful information. Aliquots of transformed cell DNA were restricted with either Hpa II or Msp I alone or with a second enzyme, Hind III. The DNA fragments were characterized by Southern blot analysis using a human β globin gene specific probe

Major Findings:

1. Analysis of total cellular RNA from two lines indicated molecules containing human β globin mRNA sequences were present at less than 100 copies per cell. Nearly complete protection of the human β globin "cDNA" probe indicated

that there is fairly uniform transcription of the coding sequences of the human β globin gene.

2. Cytosine residues, one upstream from the δ, one between the δ and β globin gene, and one downstream from the β globin gene could be examined with the two isoschizomers, Hpa II and Msp I. These sites were completely unmethylated.

Proposed Course of Project:

Despite the high frequency of co-transformation and, therefore, successful integration of the human globin genes, the low level of their expression and the apparent incorrect initiation of transcription are discouraging with respect to the potential use of this system for analysis of globin gene regulation. Other approaches are being considered whereby it may be possible to integrate defined DNA segments into mammalian cells. An effort must be made to expand the size of the DNA fragment introduced to increase the possibility that all DNA sequences necessary for normal expression will be included. A further goal is the introduction of such DNA fragments into erythroid cells, specifically the mouse erythroleukemia cell line, for in such cells normal regulation of globin gene expression could be more adequately studied than in fibroblasts.

Publications:

1. Chen, M.-J., and Nienhuis, A.W. Structure and expression of human globin genes introduced into mouse fibroblasts. J. Biol. Chem. In Press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02307 02 CHB

PERIOD COVERED
October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)
Use of Viral Regulatory Sequences to Facilitate Gene Transfer and Analysis of Gene Function

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

PI:	Keith Humphries	Visiting Associate	CHB NHLBI
	Timothy Ley	Clinical Associate	CHB NHLBI
	Arthur W. Nienhuis	Branch Chief	CHB NHLBI
Other:	Valerie P. Setlow	Staff Fellow	CHB NHLBI
	Patricia Turner	Medical Technologist	CHB NHLBI
	Austine Davis	Chemist	CHB NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH Bethesda, MD 20205

TOTAL MANYEARS: 2.5	PROFESSIONAL: 1.5	OTHER: 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)
These studies are directed toward several goals. The first is to identify and utilize positive selectable marker genes which may be used to facilitate DNA transfer into mammalian cells. The second is to compare the efficiency of hybrid viruses containing the marker gene to that of DNA introduced by the calcium phosphate precipitate technique in transforming tissue culture or hematopoietic progenitor cells *in vitro*. The third is to use viral transcription and/or replication signals to construct a DNA fragment which contains coding portions of a globin gene and which results in substantial globin production in bone marrow cells. A fourth goal is to utilize vectors containing portions of the SV-40 genome to study the function of normal and defective human globin genes in cultured cells.

PHS-6040
(Rev. 2-81)

Objectives:

The goal of correcting human genetic defects which result in severe disease by genetic therapy is becoming an increasingly more realistic objective based on substantial knowledge about gene structure and function derived from exploitation of the molecular cloning and recombinant DNA technology. Needed are positive selective markers which can be used to identify and amplify those cells into which gene transfer has been effected. We hope to utilize the gene dihydrofolate reductase, an enzyme which confers resistance to methotrexate, for this purpose. Viral DNA fragments will be utilized to provide necessary replication and transcription signals. Because SV-40 virus has been thoroughly characterized and is already used as a vector to introduce genes into eukaryotic cells, we have chosen this virus for our initial experiments. Our hope is to construct an active DHFR gene which can be packaged into SV-40 coat proteins providing a hybrid virus which can be used to infect cells thereby inducing a DHFR gene.

We have constructed and are utilizing a plasmid vector which replicates both in E. coli and in mammalian cells. This vector contains that portion of the SV-40 genome which is required for DNA replication. When this vector is introduced into a line of monkey kidney cells which constitutively produces SV-40 T antigen, amplification of the vector sequences occurs. The vector is presumably packaged into nucleosomes forming a mini-chromosome which is equivalent to the natural template for RNA synthesis. This system is being utilized to study the function of the human δ and β globin genes and also will be used to study the function of abnormal globin genes isolated from patients with homozygous β thalassemia.

Methods:

1. Construction of potentially functional DHFR genes: All constructions are performed by use of the plasmid vector, pBR322, to allow cloning of constructs into E. coli. Restriction endonuclease fragments derived from SV-40 or cloned sheep or human globin genes, are put in what is hoped to be an appropriate position with respect to the DHFR enzyme coding sequences to provide a promoter, RNA splice signals, and a polyadenylation-transcription termination complex. Individual fragments are inserted successively into a recombinant plasmid by ligation and then are introduced into E. coli by transformation; ultimately the desired construct is obtained.

2. Construction of hybrid SV-40 viral genomes: The principal for these constructions is the same as that outlined above except that the initial recombinant plasmid contains a deletion mutant of either the early or late region of SV-40. The DHFR gene is then inserted into the deleted region by recombination and molecular cloning. A viral genome containing an alternate dominant selectable marker, Eco GPT is being constructed by similar techniques. The recombinant viral genome is released from the plasmid vector by restriction endonuclease digestion and recircularized by incubation with DNA ligase.

3. Lytic infection of monkey kidney cells: Hybrid viral DNA is mixed with DNA from a termperature sensitive complementing helper virus which is defective in either the early or late region. The mixture is introduced into permissive monkey kidney cells. Following complete lysis of the cells, a viral stock is harvested which may be then used in high titer to infect permissive monkey kidney

cells. After approximately 40 hours and before cell lysis occurs, the cells are harvested and used for DNA or RNA extraction or preparation of an extract appropriate for enzyme assay.

4. Transformation of tissue culture or bone marrow cells with DNA: DNA containing the gene or genes of interest is mixed with carrier salmon sperm DNA and precipitated by incubation in a solution containing calcium phosphate. The precipitated DNA is added to cells which are then incubated at 37° for 4-24 hours. After the DNA precipitate is removed, the cells are either incubated in vitro or, in the case of bone marrow cells, reinjected into lethally irradiated mice. Selection for transformants either in vivo or in vitro is by challenge with methotrexate.

5. Characterization of transformed cells: DNA from transformed cells is analyzed by restriction endonuclease digestion, gel electrophoresis, and Southern blotting. RNA is annealed in solution to probes specific for DHFR or globin mRNA sequences to allow their quantitation. Gel electrophoresis and Northern blot analysis of RNA is used to define the size of specific RNA species. An assay for DHFR quantitates conversion of [^{3}H]dihydrofolate to [^{3}H]tetrahydrofolate providing a measure of enzyme activity.

6. Development of an SV-40 based system to study globin gene function: The standard plasmid vector, pBR322, has been modified by recombinant DNA techniques to remove a portion which inhibits its replication in mammalian cells and the SV-40 origin of replication has been inserted. The human δ and β globin genes and mutants of the latter have been inserted into this vector. Such recombinants have been introduced into Cos cells (monkey kidney cell line which constitutively produce T antigen) by the calcium phosphate mediated DNA transfer technique. DNA and RNA is recovered after 48 hours by standard techniques and analyzed electrophoretically and by hybridization.

Results:

1. Hybrid virions containing the DHFR gene in either the early or late region have been constructed and tested for their ability to confer resistance to methotrexate. These viruses were tested during lytic infection of permissive cells; the predicted species of mRNA were formed and DHFR activity in cell extracts was several fold higher than in extracts from control cells. When utilized directly, no methotrexate resistant colonies of cells were obtained. An artificially constructed DHFR gene was introduced by co-transformation with the Herpes Simplex thymidine kinase (TK) gene into TK^{-} L cells. Clones of cells were obtained which contained 10-20 copies of the DHFR gene. Nonetheless such cells did not exhibit an increased resistance to methotrexate. The mRNA produced by the artificially constructed gene apparently is translated poorly and thus does not result in a significant increase in DHFR activity.

2. Several attempts were made to transform mouse hematopoietic progenitors with either the Herpes Simplex TK gene, a mutant Chinese Hamster genomic DHFR gene, or the artificially constructed DHFR genes with SV-40 transcriptional controls. Selection for transformed cells in vivo required administration of methotrexate three times a week. In contrast to published data by other workers, we

found no evidence for DNA uptake by stem cells leading us to abandon this approach.

3. The function of the human δ and β globin genes has been studied in Cos cells. The relative expression of these genes in this test system is similar to that observed *in vivo* (β 25 x > than δ). Modification of these genes by recombinant DNA techniques should allow definitive identification of those sequences which are responsible for their different levels of expression. A relatively high level of aberrant transcription initiation or termination of globin genes apparently occurs in the Cos cells. We are utilizing a short repeated segment of the SV-40 genome to construct various recombinants which are designed to test the hypothesis that nucleosome phasing on the globin genes may be critical for correct transcription.

Significance to Biomedical Research in the Institute Program:

Many serious human diseases arise because of monogenetic mutations affecting a structural or enzymatic component within cells. Two of these in which we are particularly interested are sickle cell anemia and homozygous β-thalassemia. The goal of achieving genetic therapy for these conditions seems realistic given our current level of knowledge. In the course of pursuing this objective, additional knowledge will be gained regarding the function of transcriptional regulatory signals in eukaryotic cells.

Proposed Course of the Project:

At the present time we have several immediate goals.

1. We hope to fully develop the Cos cell system to define the genetic elements which are essential for correct globin gene transcription. This system will also be utilized to study thalassemic globin genes.

2. A hybrid virus stock containing the dominant selectable marker, Eco GPT, is being constructed. It will be used to compare the relative efficiency of virus vs calcium phosphate mediated gene transfer in a variety of cells.

3. Our artificially constructed DHFR genes will be modified in an effort to increase their function. These will be compared directly with similar DHFR constructs generated by others.

4. We plan to begin initial experiments designed to explore the potential role of hybrid adenovirus in facilitating gene transfer *in vitro* and *in vivo*.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02308 02 CHB

PERIOD COVERED
October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)
Characterization of a Repeated Sequence and Search for Polymorphisms, in Human DNA

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

PI:	Jesse Adams	Research Associate	CHB NHLBI
Other:	Amanda Cline	Chemist	CHB NHLBI
	Arthur W. Nienhuis	Branch Chief	CHB NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 2.0	PROFESSIONAL: 1.0	OTHER: 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)

An unusually long repeated DNA sequence was found 3' to the β globin gene and at 3000-4800 other sites elsewhere in the human genome. DNA sequence analysis has shown that the copy of the 6.4 repeat family abuts exactly against an Alu I repeated sequence element. Further DNA sequencing is being done to look for other examples of such opposition. A library of recombinant M13 bacteriophage containing the human genome is being screened for DNA probes capable of detecting highly polymorphic sites in the human genome which could be useful in prenatal diagnosis of genetic diseases.

PHS-6040
(Rev. 2-81)

Objectives

Repeated DNA sequences have been postulated to be related to the transposable elements which are found in prokaryotes and lower eukaryotes. Transposable elements in these species may mediate DNA mutations including deletion, and rearrangement, and may play a similar role in human DNA precipitating both genetic and neoplastic illnesses. We are studying the DNA sequence of a family of human repeated sequences in an attempt to understand the mechanism of generation of this sequence family.

It is possible to obtain a fetal DNA sample at very low risk by the technique of amniocentesis. If a panel of DNA probes were available which recognized highly polymorphic loci dispersed throughout the genome, it would be theoretically possible, through family studies, to diagnose in utero any inherited disease transmitted by Mendelian genetics. We are looking for polymorphic DNA probes which could serve this purpose.

Methods:

1) DNA sequencing: DNA fragments from different members of the 6.4 kb repeated sequence family were subcloned into the plasmid vector, pBR322, and DNA sequencing was performed using both the Maxam-Gilbert and M13-dideoxy standard DNA sequencing techniques.

2) Use of bacteriophage to prepare radiolabeled DNA probes: Human genomic DNA was digested with Sau 3A and cloned in a "shotgun" fashion into Bam H1-cut M13. Individual recombinant plaques were expanded and the single-stranded bacteriophage DNA was extracted. These template DNAs were each incubated with DNA polymerase I, radioactive [α^{32}P]dCTP and [α^{32}P]dGTP, nonradioactive, in commercially available primer, to synthesize radiolabeled DNA probes. These probes were hybridized to Southern blots of Eco RI-digested genomic DNA of various individuals in order to identify probes which hybridized with a polymorphic pattern.

Major Findings:

1) Approximately three kb 3' to the human β globin gene an Alu I repeated sequence element abuts directly against the 5' end of the 6.4 kb repeated sequence, separated only by a short string of guanosines.

2) The 5' end of another example of the 6.4 kb sequence was determined, and does not abut against an Alu I element.

3) M13 bacteriophage can be used to synthesize DNA probes of high specific activity much more rapidly than is possible using standard techniques of propagation in plasmid vectors and nick-translation.

Significance to Biomedical Research in the Institute Program:

DNA in the human genome does not code for proteins, and has no known function; the properties of this DNA are completely unknown. Our hope is that a better understanding of this DNA will enhance further our knowledge of human molecular genetics,

and of the study of genetic and neoplastic diseases in particular.

The search for DNA probes which will identify highly polymorphic loci has been initiated by other groups. We believe that we can greatly accelerate this work though by our adaptation of the M13 bacteriophage as a vector for preparing the DNA probes. Highly polymorphic probes, if identified in sufficient number, would have enormous potential for prenatal diagnosis and, thereby, possible elimination of some genetic diseases.

Proposed Course of the Project:

We hope to complete, within a few months, DNA sequence analysis of the 5' end of another copy of the 6.4 kb repeat which is close to, and may abut exactly against an Alu element. We have screened 70 DNA probes for polymorphic hybridization patterns, and plan to continue this search until we have identified several. These probes would then be studied in greater detail by hybridizing them against DNA from members of carefully pedigreed families, and then trying to identify any polymorphisms that were transmitted in linkage with known genetic defects or other phenotype markers.

Publications:

1. Kaufman, R.E., Kretschmer, P.J., Adams, J.W., Coon, H.C., Anderson, W.F. and Nienhuis, A.W. Cloning and characterization of DNA sequences surrounding the human γ, δ and β globin genes. Proc. Natl. Acad. Sci., 77:4229-4233, 1980.

2. Adams, J.W., Kaufman, R.E., Kretschmer, P.J., Harrison, M., Nienhuis, A.W. A family of long reiterated DNA sequences, one copy of which is next to the human β globin gene. Nuc. Acid Res. Vol. 8:6113-6128, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02309 02 CHB

PERIOD COVERED

October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)

Use of Hybridoma Technology in the Study of Erythroid Differentiation

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

PI:	Neal S. Young	Expert	CHB NHLBI
Other:	Sheam-Pey H. Chen	Visiting Fellow	CHB NHLBI
	Keith Humphries	Visiting Associate	CHB NHLBI
	Pedro Gascon	Staff Fellow (Clin.Assoc.)	CHB NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

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)

Monoclonal antibodies produced by hybridoma lymphocytes can have high specificity for cell membrane antigens present on distinctive cell types. Hybridoma technology has been employed in our laboratory to produce antibodies which bind selectively to hematopoietic progenitor cells. Because these cells are unavailable in quantities sufficient for immunization, the K562 leukemia cell has been employed as an antigen because its properties are similar to those of the true hematopoietic stem cell. Antibodies raised in mice against K562 cells show a variety of binding patterns to normal cells, as assayed by various immunological methods. A small number of these antibodies are inhibitory to hematopoietic colony formation under conditions which suggest their direct binding to hematopoietic progenitors. Anti-K562 cell antibodies may prove useful in disease classification, the detection of stem cells by immunologic characteristics, and the fractionation of bone marrow cells by fluorescent cell sorting. The same methods are being applied to the production of monoclonal antibodies to mouse cells in an attempt to isolate the murine stem cell by immunological means, and human-mouse lymphocyte hybridomas are being produced to isolate and characterize pathologic antibodies which occur in hematologic disease.

PHS-6040
(Rev. 2-81)

Objectives:

Antibodies produced by hybridoma technology have two important advantages over conventional antisera. First, large quantities of monoclonal antibodies can be produced because the hybridoma cell lines are "immortal". Second, the procedure for choosing the interesting antibody-producing clones allows the selection of unusual or rarely represented specificities, which would ordinarily be obscured in an analysis of an antiserum. The more readily available K562 cell has been used as an immunogen because it is likely that it shares antigenic determinants with stem cells. For example, the hematopoietic colony assay allows for the selection of antibodies which bind to hematopoietic progenitors. Thus this method appears particularly suitable for the production of antibodies to the rarely represented hematopoietic stem cell and the related hematopoietic progenitor cells that are responsible for colony formation in vitro.

As conventional methods of cell separation allow only minimal enrichment of bone marrow for hematopoietic stem cells and progenitors, purified populations of these cells cannot be employed as immunizing agents. The K562 cell line is one of three myelogenous leukemia lines capable of sustained growth in tissue culture. In contrast to other leukemia cell lines, the K562 cell expresses not only granulocytic but also erythroid, megakaryocytic, and lymphocytic cell characteristics. For example, the K562 cell may be induced to produce embryonic and fetal hemoglobins and to form small erythroid-like colonies in semi-solid media. The combined properties of "pluripotency" and the capacity for indefinite self-renewal are analogous to those of the hematopoietic stem cell. The K562 cell was chosen for immunization of mice in an effort to produce antibodies against hematopoietic stem cells present in human bone marrow.

Methods:

Balb/c mice were immunized and potent antisera produced against K562 cells. Hybridomas were prepared by fusion in the presence of polyethylene glycol of spleen cells from these animals and SP2 cells, a non-secreting mouse myeloma cell. Anti-K562 cell antibodies were screened in a radioimmunoassay using glutaraldehyde-fixed K562 cells as antigens; in some studies, normal peripheral blood lymphocytes were also used an antigens in an effort to select against antigens with broad representation on normal human cell types. Binding of anti-K562 cell antibodies to normal and malignant human cell types was assayed using a variety of immunological techniques, including complement mediated cytotoxicity, microscopic immunofluorescence, and fluorescent activated cell sorting. The binding of anti-K562 cell antibodies to progenitors was assessed by their ability to inhibit hematopoietic colony formation in vitro. Bone marrow cells, monoclonal antibody, and rabbit or human serum as a source of complement were incubated together; excess antibody was removed by washing and the cells were plated in methylcellulose in the presence of appropriate growth factors to promote colony growth. Immunoprecipitation of immune complexes formed between K562 cell membrane components and monoclonal antibodies were done using ^{125}I lactoperoxidase-labelled K562 cell membranes, solubilized in NP40 detergent, followed by separation of immune complexes by Staphylococcal A binding or anti-mouse immunoglobulin-Sepharose affinity chromatography.

Results:

Nineteen monoclonal antibodies were obtained from three separate hybridizations of animal spleens and SP2 cells. As analyzed by radioimmunoassay with a variety of normal human cells, three general binding patterns were observed. First, some antibodies bound to K562 cells and fetal or adult erythrocytes; these presumably recognized erythrocyte antigens shared by K562 cell and normal red cells, possibly including glycophorin, i antigen, and other blood group substances. Second, some antibodies bound to peripheral blood B and T cells, monocytes, and mature polymorphonuclear cells as well as to K562 cells. Finally, a third group of antibodies appeared to have relative specificity for K562 cells compared to normal cells. The binding specificity patterns have been confirmed by comparison of complement mediated cytotoxicity of K562 cells and normal bone marrow cells and peripheral blood lymphocytes. Some antibodies which have demonstrated binding to bone marrow cells have been shown to bind specifically to myeloid cells in bone marrow by microscopic immunofluorescence. The intensity of fluorescence and the number of cells with bright fluorescence in normal bone marrow have been quantitated using the fluorescent activated cell sorter. For example, antibody 76/59, which has broad specificity for lymphocytes as well as K562 cells, showed staining of myeloid cells in the bone marrow by microscopic immunofluorescence and binding to 44% of bone marrow cells by fluorescent activated cell sorting analysis. In contrast, antibody 81/57, which is more highly specific to K562 cells, showed no significant immunofluorescence to bone marrow cells and binds to only 3% or less of bone marrow cells by FACS analysis.

The binding of anti-K562 cell antibodies to hematopoietic progenitors was assessed using hematopoietic colony formation. Of ten hybridoma supernatants tested, four consistently inhibited the formation of colonies derived from the myeloid progenitor, CFU-C. A single monoclonal antibody inhibited the growth of erythroid colonies derived from the early progenitor, the BFU-E. Inhibitory antibodies included anti-K562 antibodies with both broad and narrow cell specifities. The conditions of antibody inhibition and colony growth made it likely that antibody binding to stem cells rather than to helper cells was responsible for these results.

The binding of anti-K562 cell antibodies to malignant cells has also been examined. Selected antibodies have shown binding to other myelogenous leukemia cell lines including HL-60 and KG-1 leukemic cells, which may be induced to granulocytic differentiation. In addition, these antibodies bind to myeloblasts from patients with acute myelogenous leukemia, but not to blast cells of acute lymphoblastic leukemia. Finally, several of the antibodies have shown surprisingly high binding to malignant B cells, but not to normal B cells. This binding may be related to the transformed quality of B cells treated with Epstein-Barr virus.

Proposed Course of the Project:

Attempts to identify the cell membrane constituents which are the antigens recognized by anti-K562 cell antibodies are in progress. Cell membranes of K562 and HL-60 cells have been solubilized with NP40, F_c receptors removed, and the remaining membrane components reacted with monoclonal antibodies derived from hybridoma supernatants or ascitic fluid. Immune complexes may be separated by their binding to Staphylococcus A bacteria or to anti-mouse IgG-Sepharose. Isolated immune complexes may be dissociated and the radioactively labelled antigens separated

and sized by SDS polyacrylamide gel electrophoresis.

Antibodies such as 81/57 may be useful in the preparation of bone marrow cell fractions enriched for hematopoietic progenitors or true stem cells using fluorescent activated cell sorting. This method permits a subpopulation of bone marrow cells which bind to antibody to be isolated under sterile conditions. Antigen positive and negative cell populations may be compared for the presence of colony forming units.

Similar methods are being applied to hematopoietic cells of the mouse. The murine hematopoietic stem cell can be directly assayed by the spleen colony forming unit assay. To measure CFU-S, irradiated mice are injected with spleen or bone marrow cells. The donors develop macroscopic colonies in their spleens that are the result of proliferation of a single stem cell. For monoclonal antibody studies, the Friend erythroleukemia cell has been considered equivalent to the human K562 cell and employed as an antigen. Hybridizations have been performed and anti-Friend cell antibodies identified and cloned. Antibodies which also bind to mouse bone marrow cells will be assayed for their ability to inhibit the formation of colonies derived from CFU-S.

Many collaborative studies have been undertaken to exploit the unusual properties of anti-K562 cell antibodies. In progress is an analysis of a large number of peripheral blood samples obtained from patients with acute myelogenous and lymphocytic leukemias by Dr. Ken Foon of the Frederick Cancer Research Center. Binding to selected anti-K562 cell antibodies will be correlated with clinical and laboratory characteristics, including the response to chemotherapy. With Dr. Dean Mann of the National Cancer Institute, families of patients with a variety of malignancies are being examined for antigenic characteristics present on peripheral blood lymphocytes using the fluorescent activated cell sorter. Finally, the binding of antibodies to transformed and malignant B cells is being explored in collaboration with Dr. Ian Magrath of the National Cancer Institute, who has provided a large number of cloned cell lines.

Publications:

1. Young, N.S. and Hwang-Chen, S.P.: Anti-K562 cell monoclonal antibodies recognize hematopoietic progenitors. Proc. Nat. Acad. Sci. USA, 1981, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02310 01 CHB

PERIOD COVERED October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Characterization of the Gene for Human Dihydrofolate Reductase in Normal Cells and in Cells Resistant to Methotrexate

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

PI:	M.J. Chen	Senior Staff Fellow	CHB NHLBI
	K. Cowan	Clinical Associate	MB NCI
OTHER:	A.W. Nienhuis	Branch Chief	CHB NHLBI

COOPERATING UNITS (if any)
Medicine Branch, Division of Cancer Treatment, National Cancer Institute

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.0	PROFESSIONAL: 1.0	OTHER: 0

CHECK APPROPRIATE BOX(ES)
☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Dihydrofolate reductase (DHFR) is an enzyme which is essential for cell survival and thus its gene is constituitively expressed. This enzyme is inhibited by the cancer chemotherapeutic agent, methotrexate. Cell lines, derived from human breast carcinomas have been obtained which are resistant to methotrexate. Amplification of the DHFR gene has been demonstrated in these cells by Southern blot analysis and such cells have been shown to contain an increased concentration of the mRNA for DHFR. A portion of the normal DHFR gene has been obtained as a clone from a human genomic DNA library of recombinant bacteriophage. Mapping of the 14.5 kb DNA fragment in this clone as to the location of DHFR coding sequences has been completed and the nucleotide sequence of relevant portions are being determined. A genomic DNA library has also been constructed using DNA derived from one of the methotrexate resistant cell lines.

PHS-6040
(Rev. 2-81)

Objectives:

Because dihydrofolate reductase (DHFR) has an essential role in cell metabolism and thus is constituitively expressed, it may be properly compared to specialized genes which are selectively expressed in a restricted populations of cell, e.g. the globin genes. Furthermore amplification of the DHFR gene occurs when cells are exposed to an inhibitor of this enzyme, methotrexate. The mechanism by which gene amplification occurs is unknown. Dr. Ken Cowan, a clinical associate in the NCI has developed a number of human cell lines in which the DHFR gene is amplified. As a guest worker in the CHB, he has begun to characterize these cells using the techniques of molecular biology. His presence in the laboratory has stimulated an interest in DHFR gene as a prototype for a constituitively expressed gene. Our plan is to compare the DHFR gene to globin genes with respect to transcription signals and possibly with respect to chromatin structure. Because of its ability to undergo amplification, the DHFR gene is of intrinsic interest. By comparing the structure of the natural and amplified DHFR gene in human cells we hope to deduce the mechanism of gene amplification. Finally, we are interested in using DHFR as a dominant selective marker for gene transfer into eukaryotic cells (see project "Use of Viral Regulatory Sequences to Facilitate Gene Transfer and Analysis of Gene Function"). Perhaps the natural transcription signals of the DHFR gene will be helpful in constructing a useful selectable marker gene in a viral vector.

Methods:

1. Study of gene structure: DNA is extracted from normal tissues or cell lines, digested with various restriction endonucleases, resolved on agarose gels, transferred to nitrocellulose paper, and annealed to a probe specific for DHFR sequences.

2. Molecular cloning: Genomic libraries are constructed using DNA extracted from various cell lines. The DNA is partially digested with the enzyme Eco RI and ligated to the arms of the vector, Charon 4A. In vitro packaging yields a library of recombinant bacteriophage which is introduced into a strain of E. Coli. Screening for clones containing the DHFR sequences is by the standard plaque hybridization assay or by the mini-plasmid cloning technique. (see project "Molecular Defect in β Thalassemia").

3. DNA sequencing: DNA fragments derived from recombinant bacteriophage containing DHFR gene sequences are subcloned into the plasmid vector, pBR322. Appropriate fragments are selected for sequencing. These are end labeled and a family of DNA molecules differing in length by single nucleotides is generated by the standard Maxim and Gilbert chemical degradation procedure. Resolution of these on thin polyacrylamide gels in urea followed by radioautography allows the sequence to be determined. Alternatively, fragments are cloned into the single stranded bacteriophage M13 and dideoy nucleotidesused to generate a family of DNA molecules to be resolved on polyacrylamide gels.

4. Analysis of mRNA: RNA is recovered from various cell lines by phenol extraction or by centrifugation on $CsCl_2$ buoyant density gradients. Following selection of the polyadenylated RNA species by chromatography on oligo-dT cellulose, the RNA is resolved on an agarose gel containing the denaturing agent-methyl mercury. The RNA in the gel is transferred to diazotized cellulose paper and annealed

to a probe specific for DHFR sequences. By this strategy, the size of molecular species which encode for DHFR and their relative concentrations can be deduced.

Major Findings:

1. DHFR gene in resistant human tumor cell lines: Using Southern blot analysis, the DHFR genes in several tumor cell lines has been shown to be amplified 5-10 fold. Furthermore, two of the four major DNA fragments generated by Eco RI digestion of resistant cell line DNA migrated with different mobility than did fragments from DNA of control cells. These results suggest that the length of DNA which is amplified may be relatively small.

2. Cloning of DHFR genes: A DNA fragment library constructed with normal genomic DNA has been screened by the plaque hybridization technique and a single clone containing DHFR sequences was identified. A complete restriction endonuclease map of this clone has been deduced using standard techniques and the DHFR coding sequences have been localized. The relevant portions have been subcloned into the plasmid, pBR322, and the DNA sequences are being obtained. In addition, DNA from a methotrexate resistant cell line has been cloned into the bacteriophage, Charon 4A yielding a genomic DNA library in which the DHFR sequences are amplified.

3. DHFR mRNA levels in methotrexate resistant cells: The Northern blot procedure has been used to characterize mRNA species containing encoding DHFR. An approximate 30 fold increase in concentration of DHFR sequences in resistant cell lines has been found compared to control cells.

Significance to Biomedical Research and the Program in the Institute:

This project is designed to increase our understanding of transcriptional signals which operate at the DNA sequence level in modulating gene expression. The phenomenon of gene amplification which, if understood and applied, might have important implications for genetic engineering of eukaryotic cells. Furthermore, the DHFR gene is one of the few available potential dominant selectable markers for use in gene transfer into eukaryotic cells.

Proposed Course of the Project:

Our immediate goals are straight forward; we plan to characterize the DHFR gene in normal cells and cells in which it is amplified by molecular cloning, restriction endonuclease mapping, and DNA sequencing. Several months or perhaps the next year will be required to achieve these goals. Subsequent studies will focus on the mode of amplification of the DHFR gene. Transcriptional signals of the normal and/or amplified gene may be used to construct a functional DHFR gene in viral vectors. These vectors may be useful in facilitating our general objective of gene transfer into normal eukaryotic hematopoietic stem and progenitor cells *in vitro* and *in vivo*.

Publications:

None.

ANNUAL REPORT OF THE
LABORATORY OF EXPERIMENTAL ATHEROSCLEROSIS
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1980 through September 30, 1981

Atherosclerotic lesions consist of localized thickenings of blood vessel walls. These lesions result in narrowing of the vascular lumen, which with advanced disease, may compromise blood flow. Because cholesterol contributes significantly to the mass effect of lesions, we have initiated studies to determine the mechanisms by which cholesterol accumulates within lesions. We are using human atherosclerotic vascular tissue, experimental animal models, and cultured cell systems in this work.

While previous biochemical studies by other investigators have established that both esterified and non-esterified cholesterol accumulate within lesions, analysis of whole lesions gives no indication as to the relationship of these two chemical forms of cholesterol to one another or to other lesion elements. An initial objective in our work has been to determine more precisely where cholesterol, both esterified and non-esterified, occurs within atherosclerotic lesions. We have developed histochemical techniques to study this question.

Traditional lipid staining techniques, such as oil red O, detect esterified but not non-esterified cholesterol. Using a fluorescent probe, filipin, to stain non-esterified cholesterol, we have used a combined oil red O and filipin procedure to identify microscopically both non-esterified and esterified cholesterol simultaneously in a tissue section.

Using this techinique, we have observed extracellular non-esterified cholesterol in atherosclerotic lesions of swine and human arteries. Interestingly, non-esterified cholesterol deposits occur in foci separate and distinct from extracellular cholesterol esters. Another new finding has been the observation of cells which contain only non-esterified cholesterol. These cells are distinct from classical "foam cells" which contain predominantly cholesterol esters. These observations of biochemical compartmentalization of cholesterol must be taken into account when considering cholesterol metabolism within atherosclerotic lesions. We are presently working to establish the significance of these findings.

In order to gain more information about non-esterified cholesterol deposits, we have begun studies to localize this form of cholesterol at the ultrastructural level in both atherosclerotic tissue and cultured cells exposed to lipoproteins. Future work will be directed towards identifying specific cell types within lesions and correlating their presence with cholesterol deposition.

A second program area deals with endothelial cells, which line blood vessels. It is known that certain risk factors such as high serum cholesterol or elevated blood pressure predispose to the development of atherosclerosis. It is thought by many investigators that these as well as other factors may cause injury and desquamation of endothelial cells. Injury to the endothelial lining may be a common initiating event during atherogenesis allowing passage

of lipoproteins into the vessel wall. In addition, endothelial injury induced in animal models promotes smooth muscle proliferation resulting in vessel thickening.

After vascular injury in animals, endothelial cells slough from the vessel wall and appear in peripheral blood. Based on this observation, we have begun work to assess the potential for using flow cytometry and cell sorting to quantify circulating endothelial cells in human peripheral blood. Flow cytometry is a new technology in which measurements of light scatter (i.e. cell size) and fluorescence emission are simultaneously carried out on single fluorescently stained cells as they flow in suspension past a laser and photomultiplier.

Initial studies have been successful in sorting endothelial cells based on their large light scatter signal. To increase the specificity of detection of endothelial cells from other elements with large light scatter signals, specimens will be stained with fluorescently labelled antisera against factor VIII, an endothelial cell antigen. It is hoped that the capability of quantifying very small numbers of desquamated endothelial cells circulating in peripheral blood may provide an index of vascular injury.

In conclusion, research will continue this coming year with efforts directed at understanding the mechanism of cholesterol accumulation within atherosclerotic lesions and developing noninvasive means for detection of vascular injury.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02823 02 EA

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Localization of Cholesterol in Atherosclerotic Lesions

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

	Name	Title	Affiliation
PI:	H. S. Kruth	Senior Investigator	EA NHLBI
	D. L. Fry	Professor of Medicine and Physiology Ohio State University	

COOPERATING UNITS (if any)

Section on Laboratory Animal Medicine and Surgery, NHLBI
Pathological Anatomy Department, Clinical Center

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION
Vascular Physiology

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.6	.6	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)

Cholesterol contributes significantly to the mass of advanced atherosclerotic lesions. While biochemical analysis has established that both non-esterified and esterified cholesterol occur within lesions, analysis of whole lesions gives no indication of the relationship of these two forms of cholesterol to one another or to other lesion elements. Using different histochemical probes for non-esterified and esterified cholesterol, this project has established that these two cholesterol forms occupy discrete foci within lesions. This discrete localization of non-esterified and esterified cholesterol must be taken into account in any consideration of the mechanism of cholesterol deposition, or cholesterol efflux from atherosclerotic lesions.

PHS-6040
(Rev. 2-81)

Objective: To localize non-esterified and esterified cholesterol within athero-sclerotic lesions.

Methods: Traditional lipid staining techniques such as oil red O detect esterified but not non-esterified cholesterol. A fluorescent probe, filipin, has been used to stain non-esterified cholesterol in histologic sections of human and swine arteries. A combined oil red O and filipin staining procedure has been developed in this work to identify both non-esterified and esterified cholesterol simultaneously in histologic sections.

Major Findings: Using this histologic technique, a number of new observations have been made concerning non-esterified cholesterol localization in atherosclerotic lesions. Most notably, discrete extracellular non-esterified cholesterol deposits occur and are distinct from extracellular cholesterol ester containing regions. Extracellular non-esterified cholesterol exists in three forms: as amorphous deposits, droplets, and non-birefringent and birefringent crystals. In addition to cholesterol ester containing "foam" cells, cells containing only non-esterified cholesterol also occur. These non-esterified cholesterol containing cells appear as either round (possibly monocyte) or spindle-shaped (possibly smooth muscle) cells.

Significance: The observation that distinct regions of non-esterified and esterified cholesterol exist within atherosclerotic lesions suggests that discrete metabolic events perhaps mediated by specific cells may occur during atherogenesis. More generally, these findings of extensive biochemical compartmentalization must be considered in any analysis of cholesterol metabolism within atherosclerotic lesions.

Proposed Course: Spontaneous atherosclerotic lesions from swine have been examined most extensively thus far. This investigation will now focus on human atherosclerotic lesions. In addtition, studies have been initiated to localize non-esterified cholesterol at the ultrastructural level.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02824 02 EA

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Quantification of Circulating Endothelial Cells Using Flow Cytometry

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

PI:	H. S. Kruth	Senior Investigator	EA NHLBI
Other:	R. J. Romanoff	Computer Specialist	CSL DCRT
	I. Levy	Electronics Engineer	CSL DCRT

COOPERATING UNITS (if any)

Computer Systems Laboratory, DCRT
Clinical Pathology Department, Clinical Center

LAB/BRANCH

Laboratory of Experimental Atherosclerosis

SECTION

Vascular Physiology

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 1.2	PROFESSIONAL: .2	OTHER: 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)

Endothelial cell injury is considered an important initiating event during atherogenesis. Damage to the endothelial lining of blood vessels allows passage of lipoproteins into the vessel wall. In addition, endothelial injury promotes proliferation of smooth muscle cells resulting in vessel thickening. Other investigators have described desquamated endothelial cells in blood after physical or biochemical injury to the endothelium. This project will assess the potential of using flow cytometry to quantify circulating endothelial cells in peripheral blood. Such an assay may serve as an index of vascular injury.

PHS-6040
(Rev. 2-81)

Objective: To quantify circulating endothelial cells in human peripheral blood.

Methods: A flow cytometer is being used to detect circulating endothelial cells. Human peripheral venous blood collected in EDTA is centrifuged at 395g for 20 minutes. The resulting supernatant contains platelets and endothelial cells. These cells are analyzed for light scatter (an indication of cell size) using the flow cytometer.

Major Findings: Endothelial cells lacking nuclei have been sorted from human venous blood. They can be differentiated from smaller platelets by their larger light scatter signal. However, endothelial cells cannot be differentiated from other sources of large light scatter signals such as debris. Because of the extremely low numbers of endothelial cells in peripheral blood, any other elements with large light scatter would falsely increase a count of endothelial cells. To overcome this problem, endothelial cells will be stained with fluorescently labelled antisera against factor VIII, an endothelial cell antigen. This parameter combined with light scatter should permit more specific detection of endothelial cells.

Significance: The ability to quantify desquamated circulating endothelial cells in peripheral blood may provide a noninvasive measure of vascular injury.

Proposed Course: Work will continue to improve the specificity of this endothelial cell assay. It is then proposed to screen a normal population to establish ranges for this hematologic parameter.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02825 01 EA

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Cholesterol Metabolism of Cultured Cells

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

PI: H. S. Kruth	Senior Investigator	EA NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION
Vascular Physiology

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

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

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Cultured cells take up low density lipoprotein cholesterol by two pathways, receptor and non-receptor mediated. It has been shown by other investigators that non-receptor mediated LDL cholesterol uptake in contrast to receptor mediated, does not lead to cellular cholesterol accumulation. Rather, LDL-derived cholesterol esters are hydrolyzed and excreted as non-esterified cholesterol. Type II homozygous familial hypercholesterolemic fibroblasts lack receptor-mediated uptake of LDL and thereby provide a useful model to examine isolated non-receptor mediated uptake of LDL. The purpose of this work will be to determine the fate of LDL cholesterol taken up by this pathway.

PHS-6040
(Rev. 2-81)

Objective: To examine the fate of cholesterol derived from non-receptor mediated uptake of low density lipoprotein in cultured human fibroblasts.

Methods: Non-esterified cholesterol will be localized ultrastructurally using digitonin cytochemistry. Correlative biochemical analysis of cellular non-esterified and esterified cholesterol will be carried out.

Significance: A better understanding of cellular cholesterol metabolism in cultured cell models may provide insight concerning cellular cholesterol accumulation which occurs during atherogenesis.

ANNUAL REPORT OF THE
HYPERTENSION-ENDOCRINE BRANCH
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1980 through September 30, 1981
Section on Experimental Therapeutics

This year the work of the Hypertension-Endocrine Branch has included studies of several of the major vasoactive systems: i.e., 1) renin-angiotensin-aldosterone, 2) prostaglandins, 3) sympathetic nervous system-catecholamines, and 4) kallikrein-kinin. In addition to these systems which we have studied previously, we have begun studies of a new system, the endogenous opiate peptides. Studies of all these systems and their interactions were performed to delineate the pathogenesis of hypertension and to develop better forms of therapy for this common disease.

Renin is the potent proteolytic enzyme that cleaves angiotensinogen to make angiotensin I in the initial and rate-limiting step in the production of angiotensin II, the most potent vasopressor agent in the body. The major source of renin is the juxtaglomerular apparatus in the kidney. Recently, however, there has been indirect evidence to suggest that renin might also be produced in vessel wall. Some people claim to have demonstrated that this occurs. The major problem has been that the amount of renin-like activity in blood vessels was very small and its measurement was confounded by the presence of large amounts of other proteolytic enzymes, especially the cathepsins. We have developed a method which allows us to homogenize the blood vessels, protect the renin-like activity that is present, chromatographically separate renin from cathepsins, and then assay both enzymes separately. We found that the renin in blood vessel paralleled that in the plasma, i.e., that it fell if the vessels were washed free of blood and that it fell after nephrectomy in parallel with the fall in plasma renin. Indeed, at 6 and 24 hours post-nephrectomy, no renin-like activity was detected in the blood vessels. When animals were hemorrhaged after nephrectomy in an attempt to stimulate renin formation in blood vessels, there was still no detectable renin-like activity. We were able to show that our method could recover renin if it were present and that both rat plasma renin and rat vascular renin can be inhibited by antibody against mouse renin. Thus we have strong evidence that the renin in rat blood vessels arises from plasma renin, and is not synthesized in vessel wall. Vascular renin is not stimulated by stimuli to plasma renin and there is no vascular renin in either cerebral microvessels or in aortas of nephrectomized spontaneously hypertensive rats.

We studied seven patients with aldosterone-producing adenomas before and during treatment with indomethacin to assess the role of the prostaglandins in this type of hypertension. Basal PGE_2 excretion was significantly greater than that of normals, presumably the result of the associated potassium depletion. This has been demonstrated in dogs by Stein et al. Treatment with indomethacin decreased urinary PGE_2 and thus pGE_2 synthesis by 87%, but did not affect production of aldosterone by the adenoma or of 17-hydroxycorticosteroids by the adrenal gland. Thus although prostaglandins may stimulate steroidogenesis *in vitro*, they are not essential for steroidogenesis *in vivo*. Plasma renin activity was suppressed and did not change with indomethacin. This suggests that the decrease in aldosterone with indomethacin treatment in Bartter's syndrome is probably secondary to the decrease in plasma renin activity rather than the result of an inhibition of

adrenal prostaglandin synthesis. The blood pressure of these patients with primary aldosteronism was unaffected by treatment with indomethacin and this suggests that prostaglandins have no role in this form of hypertension.

The role of the kallikrein-kinin system in renal function and in regulation of blood pressure is unknown. We have studied the changes in urinary kallikrein to acute volume expansion with various agents in unanesthetized rats. Acute expansion of extracellular fluid volume by 20% over one hour with either 0.9% saline or 5% D/W or by an equivalent amount of 3% saline produced the expected changes in urinary volume and sodium excretion, but none of these correlated with changes in urinary kallikrein. In fact, additional groups of animals were given acute volume expansion as above, and the diuresis was maintained by further infusions of the infusate in amounts needed to replace urine volume. In general, kallikrein tended to increase, but not significantly, during the hour of the acute expansion. Then it fell by the end of the hour after the infusion to a level equal to or less than the basal value. It remained relatively constant for the remainder of the study, in spite of continued diuresis or a second acute expansion with albumin. Thus the changes in kallikrein appear to represent a "washout" of kallikrein from the renal tubular cells and this is independent of excretion of volume, sodium, or potassium. The subsequent constant level of urinary kallikrein would seem to represent new synthesis and release of kallikrein by the tubular cells. This represents the most complete study of urinary kallikrein in an in vivo system and shows some of the reasons for early misinterpretations about the role of this system. We can now say that further studies of the role of kallikrein must be made in more basic systems.

We attempted to study kallikrein-like activity in a more basic system, i.e., the urinary bladder of Bufo marinus toads. However, the amount of kallikrein-like activity was very small and nearly overwhelmed by a large amount of nonspecific protease activity. Our techniques were too insensitive to note any change in the level of kallikrein-like activity when the bladders were incubated with physiological quantities of either aldosterone or vasopressin. Thus we were unable to ascertain if the level of kallikrein is determined directly in vivo by the level of aldosterone. We plan to pursue this system further when we have improved the sensitivity and specificity of our assays.

Kallikrein is physiologically inactive by itself and it has a physiologic effect only via the active vasodilator peptides, bradykinin and lysyl-bradykinin, which it produces. Some investigators have proposed that the actions of bradykinin are mediated by the prostaglandins. Therefore we observed the effects of infusion of bradykinin on renal function of dogs before and during pre-treatment with either indomethacin or propranolol. Infusion of bradykinin significantly increased urine flow, excretion of sodium and potassium, and renal plasma flow without a consistent change in glomerular filtration rate or renin secretion rate. These effects of bradykinin were unchanged by treatment with either indomethacin or propranolol. Thus the actions of bradykinin in the kidney of the dog are independent of the prostaglandins or B-adrenergic receptors.

Last year we developed a high pressure liquid chromatographic-electrochemical detector assay for plasma catecholamines. We have applied this methodology to a number of different situations to learn more about the sympathetic nervous system in circulatory regulation. Hypertensive patients have higher plasma levels of norepinephrine than normotensives. To assess the source of this

difference, we have measured catecholamine concentrations in both arterial and venous blood. In early studies, we find that hypertensives have higher arterial and venous norepinephrine levels than normotensives, as well as greater arteriovenous differences in norepinephrine. Hypertensives also have higher arterial and venous levels of epinephrine than normotensives, but no increase in the arteriovenous difference in epinephrine. The higher venous levels suggest that accentuated peripheral sympathetic and adrenal medullary activity may characterize a proportion of patients with essential hypertension. In baboons conditioned to raise their blood pressure in response to a light, the increases in systolic and diastolic blood pressure and in heart rate paralleled increases in plasma norepinephrine and epinephrine, while plasma dopamine and renin activity were unchanged. Thus, in this hypertensive model, there is a good correlation between blood pressure and sympathetic activity. We studied 12 patients with surgery-proven pheochromocytomas and 13 patients with hypertension, but without a pheochromocytoma. Measurement of plasma catecholamines appears to provide a quick and accurate means of diagnosing a pheochromocytoma when resting norepinephrine levels are more than 600 pg/ml. However, when plasma norepinephrine is less than that, the diagnosis cannot be excluded.

In an attempt to see if stress elevates blood pressure we studied 21 normal volunteers during dentally-indicated wisdom tooth extractions. The surgery increased heart rate, systolic blood pressure and cardiac output, without a significant change in diastolic blood pressure. Plasma norepinephrine increased markedly in non-sedated patients, but diazepam sedation abolished the norepinephrine response to surgery without affecting the heart rate or systolic pressure responses. Plasma epinephrine increased about three-fold with the surgery and five-fold if epinephrine was injected along with the local anesthetic. Thus the sympathetic nervous system participates actively in the responses to the stress of dental surgery. We are now extending these studies to hypertensive patients undergoing dental surgery to see how their sympathetic nervous system responses compare to normals.

One proposed mechanism for hypertension has been a defect in the baroreflex mechanism. Previous studies have divided nearly equally into those that demonstrate a defect and those that do not. The studies have used different methods, but have rarely used more than one method at a time to allow for a comparison of results.

We have now studied baroreflex sensitivity in 30 subjects by means of three different methods and eight different measurement techniques. The average intercorrelation among these measures was significant ($r=0.36$, $p<0.01$), but suggests that variance in one measure accounted for only about 13% of the variance in others. Standard deviations across subjects often were as large as the mean, indicating important inter-individual variability as well. Nevertheless when age-matched subgroups of 10 hypertensives and 7 normotensives were compared, patients with essential hypertension appeared to have diminished baroreflex sensitivity. We have also tested some patients before and after therapy with the antihypertensive drug, clonidine. This drug acts centrally to reduce sympathetic nerve output and has been said to affect baroreflex function. However, we have noted no obvious effect of clonidine on baroreflex sensitivity, in spite of 70% reductions in plasma norepinephrine. To determine if the antihypertensive actions of clonidine are mediated via the endogenous opiate peptides,

we have studied the blood pressure response to acute injections of the opiate antagonist, naloxone, given before and during therapy with clonidine. So far no effect of even large doses of naloxone on blood pressure has been seen. Thus the anti-hypertensive action of clonidine in man is not mediated by endogenous opiate peptides.

Hypertension may start as primary constriction of the resistance vessels or it may be perpetuated once it begins by secondary structural changes in the vessels. To be able to study these vascular changes in man, we have developed a new technique for measuring blood flow in the hand during maximum vasodilation. This is accomplished by use of 43°C. water, ischemia, and venous occlusion plethysmography. In addition, transmural vascular pressure was varied by increasing the hydrostatic pressure in the plethysmograph. When the different levels of transmural pressure achieved by varying the pressure in the plethysmograph were plotted against blood flow, a straight line was obtained. In early studies, mean blood flow was significantly greater in normotensives than in patients with uncomplicated essential hypertension. Thus, even these patients appear to already have structural alterations in their blood vessels. This technique fits well with our studies of baroreflex sensitivity and of arteriovenous differences in catecholamine levels. We can now evaluate when in the course of hypertension vascular changes occur, if the vascular changes are reversed by treatment of the hypertension, and which drugs are most effective in reversing the vascular changes.

Patients with Bartter's syndrome have an increase in sympathoadrenal activity as indicated by significant increases in norepinephrine and epinephrine excretion. Correction of overproduction of prostaglandins by treatment with an inhibitor of prostaglandin synthesis restores urinary norepinephrine and epinephrine to normal. To see if potassium depletion may be the basis for the increase in prostaglandins and in sympathetic nervous system activity, hypokalemia was experimentally induced in rats. The potassium-depleted rats showed an increase in plasma norepinephrine, epinephrine and renin activity. Thus, potassium depletion is a potent stimulus to the sympathetic nervous system and the action appears to be mediated by the prostaglandins. Further studies are needed to determine specifically which prostaglandins are involved.

Opiate peptides and other peptidergic neurotransmitters are found only in cells that contain a neuronal specific enolase (NSE). The adrenal medulla is especially rich in NSE so we assayed the blood and tumor tissue from a number of our patients with pheochromocytomas. We found that pheochromocytoma cells are rich in NSE, but the levels of NSE in the blood of patients with pheochromocytoma are normal. (This is very different from oat-cell carcinomas of the lung, where both the tumor and the plasma have very high levels of NSE.) Indeed, there was no correlation between plasma NSE levels and levels of the principal plasma catecholamine made by the pheochromocytoma. Thus, plasma NSE is of no value in the diagnosis of pheochromocytoma. However, of interest is the finding that plasma NSE levels appear to be higher in hypertensives than in normotensives. This finding will be followed up in age and race-matched subjects because it raises important questions about a peptidergic mechanism in essential hypertension.

Recently a role for vagal afferents in circulatory control has been postulated. We sought to test this by studying endotoxin shock in rats. Preliminary data indicate that abdominal vagotomy reverses the hypotension, prolongs life,

and improves survival in response to endotoxic shock, while treatment with atropine does not. This suggests that vagal afferents may well carry noxious stimuli that have deleterious effects on the circulation. To further test this hypothesis, we have found that the hypotensive response to exogenously administered opiate peptides in rats is considerably attenuated or abolished by subdiaphragmatic bilateral vagotomy, but not by atropine administration. We must also follow up on these findings since they have important potential ramifications.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01924-03 HE

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
The Search for Renin-Like Activity in Vessel Wall

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3.0	1.0	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)

Extra-renal vascular renin had not been clearly demonstrated partly because the levels are very low. Since acid proteases (AP), active at this pH^{6}, may both generate Angiotensin I (AI) and destroy apparent RLA, protease inhibitors and affinity chromatography were used. In aortas collected from animals 24 hours after nephrectomy and 18 hours after hemorrhage no detectable RLA was present. No RLA was found in cerebral microvessels nor was it present in the aortas of spontaneously hypertensive rats collected 24 hrs after bilateral nephrectomy. Anti-renin antibody completely inhibited rat PRA and vascular RLA. Vascular RLA appears to arise from the plasma, and extravenal vessels do not appear to be a separate source of renin.

PHS-6040
(Rev. 2-81)

Objectives: The wall of the afferent arteriole of the glomerulus has differentiated into specialized cells which produce renin. Renin is the rate-limiting enzyme that leads to the production of Angiotensin II, the potent vasoconstrictor. It has been proposed that vessels outside of the kidney may also be a source of renin. Our experiments were designed to measure renin-like activity in vascular tissue and to determine the source of the activity. To maximize detection of tissue renin, samples were incubated at pH 5.9, near the pH optimum for renin. Acid proteases such as Catepsin D, active at this pH, are capable of generating Angiotension I (AI). Protease inhibitors were used to protect the enzymes of interest. Furthermore, affinity chromatography was performed to remove acid proteases prior to incubation. And additionally, since Skeggs and others have shown that acid proteases generate AI poorly from homologous plasma substrate, nephrectomized rat plasma was used as a source of angiotensinogen.

Methods: Vascular Renin-Like Activity in Aorta. Male Sprague-Dawley rats (250-350 g) received one dose each of intramuscular furosemide (0.57 mg/kg) and then were placed on a 0.18% sodium diet for two weeks. All animals had free access to deionized water. This diet represents only mild sodium restriction (compared to the standard NIH diet of 0.45% sodium) and does not interfere with growth.

Four separate experiments were performed. In experiment I the aortas were collected from seven normal rats for the measurement of vascular renin-like activity and proteolytic activity as described below. In experiment II three subgroups each of seven rats were prepared. Aortas were collected from rats two hours after bilateral nephrectomy (subgroup I), six hours after bilateral nephrectomy (subgroup II), and 24 hours after bilateral nephrectomy (subgroup III). In experiment III six hours after bilateral nephrectomy each of seven rats was hemorrhaged 15-20% of his total blood volume. The blood volume was calculated from an estimated ratio of 50cc total blood volume/kg weight in the rat. At 24 hours after nephrectomy and eighteen hours after hemorrhage, the aortas were collected. In experiment IV aortas were collected from seven normal rats after the standard perfusion with 120 ml iced-saline (described below). A 20 gauge needle was inserted into the lumen of each aorta and the vessel was vigorously perfused with an additional 50 ml of iced saline. The vessels were then processed to measure the vascular renin-like activity and the proteolytic activity.

Two different anesthetic regimens were used to minimize any effect on experiments which resulted from the choice of anesthetic. Surgical procedures were performed under anesthesia induced with either pentobarbital or ketamine and Acepromazine. No differences were noted. After induction of anesthesia, a midline incision was made in the abdominal wall. The animal was exsanguinated and then perfused with 120 ml of iced saline to remove any residual blood. The aorta from the arch to the bifurcation of the iliacs was quickly removed and placed on ice. The adventitia was carefully dissected free and its removal was verified by light microscopic examination of several samples. The aorta was cut into rings one to two millimeters in length and frozen. The aorta would be subsequently prepared for chromatographic separation of vascular renin-like activity from proteolytic activity. Meanwhile, the plasma renin sample was immediately processed at room temperature to avoid cryoactivation. Blood was placed into sterile Vacutainer collection tubes which contained potassium-EDTA as an anticoagulant. The blood was centrifuged to remove the cellular elements, and the plasma separated and rapidly frozen. All tissue and plasma samples were stored at -196°C in liquid nitrogen.

Chromatography for Samples of Aortas. For each experiment one gram of aortas (wet weight) was used. The aortas were frozen and thawed four times. The tissue was suspended in one ml of cold column buffer pH 4.0 which consisted of .02 M acetate, 1 mM dipotassium EDTA, 1 mM EGTA, 1 mM sodium tetrathionate and 0.25 mM phenylmethylsulfonyl fluoride (PMSF). Such inhibitors have been used to protect renin from proteolytic degradation during purification procedures and do not inhibit renin activity. The tissue was first homogenized on ice with a polytron homogenizer, and then sonicated with a Kontes micro-ultrasonic cell disrupter. The 40,000 x g supernate was then concentrated to 500 μl in a minicon-B15 concentrator. The sample was placed on a column of bovine hemoglobin immobilized on Sepharose-4B previously equilibrated with column buffer. The affinity resin was bovine hemoglobin attached to CNB-activated Sepharose 4B. The flow rate through the 0.9 x 15 cm column was 1.1 ml/hr. Renin, which has no nonspecific proteolytic activity, passes through the resin with the column buffer. The acid proteases bind to the hemoglobin and can be eluted with a buffer of alkaline pH and high ionic strength (0.1 M TRIS, 1.0 M sodium chloride, 1 mM EDTA, and 1 mM EGTA). The vascular renin-like activity and the proteolytic activity were measured in each fraction.

Vascular renin-like activity in fractions of the chromatographed samples was measured with nephrectomized rat plasma, a source of angiotensinogen. Blood was processed rapidly at room temperature to avoid cryoactivation, and the plasma was quickly frozen and stored at -196°C. The baseline renin activity in each pool of substrate was measured.

In general 250 μl of sample was incubated with 750 μl of nephrectomized rat plasma in the presence of 15 μl of 15% dipotassium EDTA, 10 μl of 10% neomycin sulfate, and 5 μl of 5% PMSF. Each sample was individually adjusted to pH 5.9 and divided into two aliquots. One aliquot was incubated at 37°C for 18 hours and the other at 4°C for 18 hours. The 4°C incubation was used as a blank for the endogenous AI immunoreactivity in each sample. In addition incubations of nephrectomized plasma in the presence of either column buffer or elution buffer were performed in each experiment.

Proteolytic Assay. The proteolytic activity in each fraction was measured as the trichloroacetic acid-soluble peptide fragments of proteolytic cleavage of radioactively labeled hemoglobin substrate. Labeled substrate was obtained either as a kind gift from Dr. French Anderson or from New England Nuclear (^{14}C tagged methylated methemoglobin MEC-728). The reaction mixture consisted of 100 μl of sample, 400 μl of 0.1 M acetate pH 3.5, and 10 μl of label (25,000 cpm). The incubation proceeded at 37°C for one hour and was terminated by the addition of 300 μl of 20 mg/ml bovine hemoglobin in 0.1 M acetate buffer pH 3.5 and 500 μl of fresh 10% trichloroacetic acid. After centrifugation one ml of the supernatant is counted and is reported as cpm/hour/0.1 ml sample. Bovine spleen cathepsin D was used to test the sensitivity of the assay. Cathepsin D was serially diluted and aliquots of the dilutions were placed into the proteolytic assay and into the tissue renin assay. The proteolytic assay could detect at least one order of magnitude smaller concentration of cathepsin D than the renin assay could defect by measurement of AI generated from substrate.

Protein. Protein concentrations were estimated in fractions by measurement of the absorbance at 280 mM.

Vascular Renin-like Activity in Microvessels. Cerebral microvessels from five Sprague-Dawley male rats were prepared by a method of Mrsula et al.

Vascular Renin-Like Activity in the Spontaneously Hypertensive Rat. Seven male SHR's (350 gm) were taken from the NIH colony of spontaneously hypertensive rats (average blood pressure of 180 ± 2 mm Hg) for the measurement of aortic vascular renin-like activity. The SHR's were bilaterally nephrectomized and twenty-four hours later the aortas were collected. One gram of aortas was chromatographed as described above.

Inhibition of Vascular Renin-Like Activity by Anti-Renin Actibody. Dr. Eve Slater kindly supplied us with a rabbit antibody against mouse renin. Either anti-renin antibody or rabbit non-immune serum was incubated in a 1:1 dilution with either normal rat plasma or vascular renin-like activity collected after chromatography. The mixtures were kept at 4°C for 24 hrs. As a source of angiotensinogen nephrectomized rat plasma was added to the mixtures containing vascular renin-like activity. The endogenous angiotensinogen in normal rat plasma provided the source substrate for the plasma samples. Aliquots of the samples were incubated at either 37°C or 4°C in the presence of EDTA, neomycin and PMSF as described above. The AI was measured by radioimmunoassay and reported as ng AI/ml/hr.

Recovery of Porcine Renin. To estimate the losses of renin activity throughout the procedure .0075 Goldblatt units of porcine renin (United States Biochemical Corp., Cleveland, Ohio) were added to aortas collected from rats 24 hours after bilateral nephrectomy. The mixture was homogenized, chromatographed, and the fractions assayed in the usual manner. The recovery was calculated from the total AI generated in the chromatographed fractions and that present in the homogenate prior to chromatography.

Results: Vascular Renin-Like Activity in Aortas from Sprague-Dawley Rats.

Experiment I: Aortas from Normal Rats.

The affinity chromatogram of one gram of aortas from normal rats revealed two peaks of RLA. Peak 1 contained no proteolytic activity while peak 2 did. The peak RLA in peak 1 was greater than 460 ng AI/ml/18 hr. The RLA in peak 2 was less than 10 ng AI/ml/18 hr., small by comparison to peak 1.

Experiment 2: Aortas from Bilaterally Nephrectomized Rats.

The affinity chromatogram of one gram of aortas collected from rats two hours after bilateral nephrectomy revealed only one distinct peak of RLA which eluted with column buffer in a position comparable to that of peak 1 above. The peak RLA reached 76 ng AI/ml/18 hrs. At 6 hours and at 24 hours after bilateral nephrectomy, no detectable RLA was present. The absorbance at 280 nM was measured in these latter subgroups and demonstrated that even through no RLA was present, proteins which did not bind to the bovine hemoglobin were eluted with column buffer as expected. In all subgroups proteolytic activity was eluted with change to TRIS buffer pH 8.6.

The plasma renin activity fell from 31.4 ± 5.9 ($\bar{x}$ ± SD) ng AI/ml/hr prior to nephrectomy to 2.89 ± 1.51, 0.54 ± 0.12, 0.20 ± 0.13 ng AI/ml/hr at 2, 6, and 24 hours after nephrectomy, respectively.

Experiment III: The chromatogram of one gram of aortas collected from rats twenty-four hours after bilateral nephrectomy and eighteen hours after hemorrhage revealed no detectable RLA. Proteolytic activity eluted as expected.

Experiment IV: The additional wash of each aorta with 50 ml of iced saline reduced the peak RLA from better than 460 ng AI/ml/18 hrs. to near 10 ng AI/ml/18 hr.

Results: Microvessels

The affinity chromatogram of cerebral microvessels revealed no detectable RLA in the microvasculature.

Results: Spontaneously Hypertensive Rats

Twenty-four hours after nephrectomy no vascular RLA was present in the aortas of SHR's.

Results: Antibody Inhibition Study

Under the incubation conditions described above, rabbit antibody against mouse renin completely inhibited rat vascular renin-like activity as well as rat plasma renin activity.

Results: Recovery Experiments

The recovery of porcine renin was measured through two separate chromatographic runs. In the first the overall recovery was 28.9% and in the second the overall recovery was 34.8% for an average of 32%. Thus if one gram of aortas is used, one should see the level of renin activity present in 300 mg of aortas (three-fold the level many investigators use).

Significant Findings:

1) Renin-like activity (RLA) is present in vessels of normal rats.

2) Vascular RLA decays with the decay in plasma renin activity.

3) Vascular RLA is not stimulated by hemorrhage in the nephrectomized animal.

4) Extensive washing of the aorta decreases the measured vascular RLA.

5) No RLA is present in cerebral microvessels.

6) No vascular RLA is present in the aortas of nephrectomized SHR's.

7) Both rat plasma renin activity and rat vascular RLA can be inhibited by antibody against mouse renin.

Therefore, vascular RLA arises from the plasma. Extrarenal vessels do not appear to be a separate source of renin.

Proposal: After submission of data for publication, no plans have been made to pursue this model further. The techniques can be used to investigate renin activity in a variety of organs including the central nervous system.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01929-02 HE

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

The Sympathetic Nervous System and Stress

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

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OTHER:	Harry R. Keiser, M.D.	Deputy Chief	HE NHLBI

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.20	.20	

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

We are measuring cardiovascular (heart rate, blood pressure, and cardiac output), biochemical (plasma norepinephrine, epinephrine, growth hormone, cortisol, cholesterol, triglyceride, and lipoprotein), and psychological (pain, anxiety) responses to wisdom tooth extraction in healthy young adults and in patients with essential hypertension.

PHS-6040
(Rev. 2-81)

Objectives: Undergoing wisdom tooth extractions is a real-life, acute emotional stress. By measuring plasma norepinephrine levels--reflecting sympathetic neural activity--and cardiovascular variables, we hope to characterize the response to non-physical stress in normal persons and patients with essential hypertension, since essential hypertensives appear to show excessive sympathetic responsiveness to stress which may have pathophysiologic significance (1).

Methods: Blood samples drawn through indwelling intravenous needles have been assayed for the above biochemical parameters a few days prior to surgery, and during the pre-operative, operative, and post-operative conditions, for each of two sets of wisdom tooth extractions. For the first operation, patients were randomly assigned to receive or not to receive sedation with intravenous diazepam, and for the second, were assigned to receive or not to receive epinephrine with the local anesthetic. Impedance cardiography has been used as a non-invasive measure of cardiac output. Experienced pain and anxiety have been measured using standardized questionnaires.

Results and their significance: Across 21 healthy patients, the surgery was associated with significantly increased heart rate (25%), systolic blood pressure (13%), and cardiac output (34%) without a significant change in diastolic blood pressure. Plasma norepinephrine increased by 60% during the surgery in non-sedated patients. Diazepam sedation abolished the norepinephrine response to the surgery, without significantly affecting the heart rate or systolic pressure responses. Receipt of epinephrine with the local anesthetic resulted in a five-fold increase in mean plasma epinephrine five minutes after the injection, as well as increased cardiac output. The direct effect of epinephrine accounted for the cardiac output increase observed during the surgery. In non-sedated patients not receiving epinephrine with the local anesthetic (N = 8), endogenous plasma epinephrine increased about three-fold with the surgery.

The results suggest the participation of the sympathetic nervous system in producing the circulatory responses to dental surgery. The elimination of sympathetic recruitment by diazepam-induced sedation, however, without concomitant reductions in heart rate of systolic pressure responses, suggests that other systems besides the sympathetic nervous system influence the circulatory response to this real-life stress. In contrast with isotonic exercise, which produces greater norepinephrine than epinephrine responses in healthy persons, the emotional stress of dental surgery produces greater epinephrine than norepinephrine responses.

Proposed course of project: The protocol has been amended to include study of patients with essential hypertension. We will need actively to recruit participation of young patients with hypertension in whom removal of wisdom teeth is clinically indicated. We anticipate studying 10 patients with essential hypertension in the next year.

Publications:

1. Goldstein, D.S.: Plasma norepinephrine during stress in essential hypertension. Hypertension. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01930-02 HE

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Baroreflex Sensitivity in Hypertension

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

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.25	.25	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

We have measured baroreflex sensitivity in patients with essential hypertension and in normotensive controls using 8 different measurement techniques, in order to determine whether the techniques agree with each other, if baroreflex sensitivity is abnormal in essential hypertension, if clonidine exerts its antihypertensive action by affecting the baroreflex, and if naloxone antagonizes clonidine's effects.

PHS-6040
(Rev. 2-81)

Objectives: Since the baroreflex is the most powerful, rapidly acting circulatory homeostatic reflex, we have investigated whether an abnormality in the functioning of this reflex characterizes patients with essential hypertension. Because results of previous studies have disagreed about whether hypertensives show abnormal reflex sensitivity, and because any of several measurement techniques have been used, we compared eight techniques in the same subjects to determine the extent of agreement among them.

We also wished to test the hypotheses that the anti-hypertensive effects of clonidine are mediated by affecting baroreflex sensitivity, and that naloxone antagonizes clonidine's anti-hypertensive effects by inhibiting endorphins.

Methods: To conduct this study, a neck cuff apparatus was specially designed and fabricated to allow delivery of rapid changes in external neck pressure. The techniques used to assess baroreflex sensitivity were the change in R-R interval per unit change in systolic pressure during the Valsalva maneuver, upon release of the Valsalva maneuver, after injection of phenylephrine, after injection of nitroglycerine; the changes in R-R interval and in systolic pressure per mm Hg externally applied neck suction; and the changes in R-R interval and systolic pressure per mm Hg externally applied neck pressure. Patients with essential hypertension, off anti-hypertensive medication for at least 2 weeks, underwent baroreflex testing, as did normotensive controls. In hypertensives, the baroreflex testing procedure was repeated after 1 week of oral therapy with clonidine.

Results and their significance: The average intercorrelation among these measures in 30 subjects was statistically significant ($r = 0.36$, $p < 0.01$), but suggests that, on average, variance in one measure accounted for only about 13% of the variance in others. Standard deviations across subjects often were as large as the mean, indicating important inter-individual variability as well. Nevertheless, when age-matched subgroups of 10 hypertensives and 7 normotensives were compared, baroreflex rankings averaged across measurement techniques were significantly poorer in the hypertensives ($t = 3.27$, $p < 0.01$). Patients with essential hypertension therefore do appear to have diminished baroreflex sensitivity.

Three hypertensive patients have undergone repeat baroreflex testing after clonidine therapy. Clonidine decreased resting venous plasma norepinephrine by over 70% in these patients, resting systolic and diastolic pressures decreased to the normotensive range, and the overshoot in systolic pressure after release of the Valsalva maneuver was markedly attenuated, all these findings being consistent with effective therapeutic levels of clonidine. However, no obvious change in baroreflex sensitivity has appeared. The data so far are inconsistent with the hypothesis that clonidine's anti-hypertensive effect derives from accentuated baroreflex sensitivity. No obvious effect of naloxone on blood pressure in clonidine-treated patients has appeared.

Proposed course of study: We wish to extend the study to allow statistical analysis of age-matched hypertensive and normotensive groups, to measure baroreflex sensitivity in patients with secondary hypertension, and re-measure baroreflex function in treated hypertensives, to determine if the reflex abnormalities are part of the cause, or merely the result, of essential hypertension.

The observations with regard to clonidine and naloxone will be extended to an adequate number of patients to perform statistical analyses. We envision that these studies will take 1 to 2 years.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01931-02 HE

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Effects of Alterations of Extra-Cellular Volume on Urinary Kallikrein Excretion

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

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OTHER:	Harry Keiser, M.D.	Deputy Chief, Hypertension-Endocrine Branch	HE NHLBI
	Marian E. Warner, BS	Biologist	HE NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 2.0	PROFESSIONAL: 1.0	OTHER: 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 role of the Kallikrein system in adaptation to acute alteration in extra-cellular volume and salt concentrations was evaluated in female Sprague- Dawley rats. Extra-cellular volume was expanded by 20% with either NS or D5W over a period of one hour, at which point the infusion was either stopped or continued to match urine output. There was no correlation between kallikrein and urinary volume, sodium, or potassium. Kallikrein excretion demonstrated a pattern suggestive of a tubular "washout" phenomenon in response to volume expansion that was unaffected by adrenalectomy.

PHS-6040
(Rev. 2-81)

Objective: A relationship between urinary excretion of kallikrein and salt handling by the kidney has been postulated for some time. Previous studies have reported conflicting results and model systems have not been standardized. This study was designed to evaluate the response of the kidney in terms of kallikrein excretion to acute volume expansion, performed in conscious animals similarly prepared and expanded. Kallikrein has also been tentatively assigned a role in blood pressure regulation; therefore its interaction with volume, salt, and the kidney is of great interest.

Methods: Female Sprague-Dawley rats weighing 200-290 grams (Taconic Farms) were randomized into nine groups of 6 to 8 animals. Each group received a separate expansion protocol as noted below. The rats were anesthetized with pentobarbital (5 mg/kg I.P.), and the right external jugular vein was cannulated and the urinary bladder catheterized. In one group adrenalectomy was performed thirty six to forty hours preceding the expansion experiment with the animals receiving dexamethasone replacement (1 μg/kg per day I.P.). The awake rats were placed in specially designed cylindrical plexiglass holders.

Urinary kallikrein was measured by a radioimmunoassay developed in the laboratory of Dr. John Pisano.

Results:

Group 1: Received 0.9% saline in D5W throughout the entire experiment at 0.6 ml/hr. These animals demonstrated stability of all measured parameters through all the collection periods. Baseline kallikrein was not significantly different among any of the non-laparotomized animals.

Groups 2: 20% expansion of ECV with 0.9% saline in one hour. Group 3: 20% expansion with D5W in one hour. In these acute 20% expansion groups there was a significant increase in urinary volume during the expansion period which persisted for at least one additional period. Sodium excretion was increased with the 0.9% saline and unchanged with dextrose and water. Potassium excretion rose during the initial dextrose infusion then fell to baseline, while with the saline, excretion was unchanged. Urinary kallikrein rose in both groups during the hour of expansion although this rise was not statistically significant. In the four hours following expansion, kallikrein excretion was significantly lower than during expansion in Group 2, and lower than baseline in Group 3. Kallikrein returned to basal levels by the seventh hour in both groups.

Group 4: 15% expansion of total body sodium (calculated as 45 meq/kg body weight) with 3% saline in one hour. Infusion with 3% saline increased urinary sodium with no change in volume. A fall in kallikrein began during the infusion period and this reached significance during the following two hours, returning to baseline by four hours. Potassium excretion was unchanged throughout the experiment.

Group 5: 20% expansion of ECV with 0.9% saline then continued replacement of urine volume to maintain a saline diuresis. Group 6: 20% expansion of ECV with D5W and continued replacement of urine volume to maintain a water diuresis. The 0.9% saline and water sustained diuresis experiments demonstrated similar patterns of increase in urinary volume, however, sodium excretion was unchanged with water diuresis and rose non-significantly during the hour of initial expansion, followed

by a fall in both groups. In Group 5 post infusion kallikrein remained significantly lower than during the primary expansion but unchanged from baseline.

Group 7: Initiation of saline diuresis with 20% ECV expansion, replacement for 4 hours of urine output with 0.9% saline, then replacement of urine output for 5 hours with a solution of 0.9% saline and 25% bovine albumin (6.5 mg/kg). This group receiving 0.9% saline with the latter addition of hyperoncotic albumin demonstrated two separate changes in urinary volume and sodium excretion. The initial expansion provided an immediate increase in the excretion of volume and sodium followed by a typical diuresis with stabilization of both variable over time. Addition of albumin produced a pronounced and significant increase in urine volume and sodium which persisted for two hours before returning to pre-albumin levels. Urinary potassium rose with expansion and remained elevated throughout with no evidence of a secondary effect from the addition of albumin. A nonstatistically significant increase in kallikrein was noted with the initial expansion followed by a fall. However, no significant changes were noted in kallikrein with the addition of albumin that corresponded with the marked changes in urine sodium and volume noted above.

Group 8: Sham operated rats with 20% ECV expansion with 0.9% saline and 3 hour collection post expansion. Group 9: Adrenalectomized rats with 20% ECV expansion with 0.9% saline and the same collection periods as Group 8. Sham and adrenalectomized rats followed the same pattern with 20% ECF expansion as did Group 2. Baseline kallikrein levels were 50% lower in the adrenalectomized group when compared to the sham controls. In both groups kallikrein was significantly lower immediately following the expansion period. No significant differences existed in sodium, volume, or potassium excretion between the two groups.

Major Findings: The control of volume is crucial for an organism's survival. Control of salt and water excretion is central to volume regulation. The kallikrein-kinin system both alone and through connection to other renal mechanisms has been postulated to play a major role in this control. For the most part these conclusions are based on data provided from experiments in animal preparations not carefully standardized. The present experiments attempt to provide answers to basic questions. These are acute studies and are limited to relatively short term changes. However the degree of expansion is well within the range of normally tolerated physiologic changes, and has been demonstrated to significantly modify measured variables in the system. The demonstration in these studies of what appears to be a "washout" effect as the etiology of the rise noted in urinary kallikrein is an important finding. It is apparent that the simple measurement of urinary kallikrein is not sufficient to evaluate the role of this system in the excretory function of the kidney, and further work must utilize a more basic approach.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01932-01 HE

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Collaborative Studies of Plasma Catecholamines in Man

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

PI:	David Goldstein, M.D., Ph.D.	Clinical Associate	HE	NHLBI
OTHER:	Harry R. Keiser, M.D.	Deputy Chief	HE	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
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Experimental Therapeutics

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NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.15	.15	

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

We are assessing the effects of amphetamine injection on blood pressure, heart rate, and plasma catecholamines in healthy volunteers, and relationships among plasma catecholamines, circulatory variables, and physical exertion in patients with hemophilia. These studies take advantage of the assay techniques developed and validated in this laboratory for measuring plasma catecholamines.

PHS-6040
(Rev. 2-81)

Objectives: Individual differences in response to amphetamine have been used as a predictor of clinical response to tricyclic antidepressants, and as such may provide a clue to the genetic vulnerability to affective disorders. This project investigates the inheritance of the behavioral and physiological effects of d-amphetamine. Since amphetamine affects sympathetic neuronal handling of norepinephrine, we wish to determine the relationship among plasma norepinephrine and blood pressure after amphetamine administration.

Exercise training improves coagulation parameters in patients with hemophilia. It is possible that this benefit derives from the mediating effect of plasma epinephrine. We therefore are measuring plasma epinephrine in patients with hemophilia during exercise to determine the relationships, if any, with coagulation parameters.

Methods: These studies are being conducted primarily at the NIMH and Children's Hospital, respectively. We are collaborating with the principal investigators because of our continuing interest in interrelationships among plasma catecholamines, circulatory parameters, and clinical disease states. In the amphetamine study, patients are infused with a single intravenous infusion of 0.3 mg/kg d-amphetamine over a 5-minute period. Blood pressure and heart rate are recorded during the subsequent 90 minutes and blood samples taken for assay of plasma catecholamines. In the hemophilia study, patients undergo bicycle ergometer exercise to pre-determined heart rate or systolic pressure criteria, and blood is drawn before, during, and after the exercise for determination of plasma catecholamines and coagulation parameters.

Results and their significance: Three patients have been studied in the amphetamine study. In all three, amphetamine administration resulted in increased blood pressure and correlated increases in plasma norepinephrine. Both blood pressure and norepinephrine began to increase within 5 minutes of the infusion and peaked at about 15 to 30 minutes. Amphetamine's effects on blood pressure therefore appear to derive from its effects on norepinephrine.

Eight healthy young adults and 10 patients with hemophilia have undergone the exercise testing procedure at Children's Hospital. Although the exercise produced predictable changes in epinephrine and norepinephrine concentrations, no correlated changes in coagulation parameters have been noted. These results tend to refute the hypothesis that plasma epinephrine influences coagulation parameters in patients with hemophilia.

Proposed course of project: These collaborative studies will be extended to allow enough patient data for statistical analyses. This should take about 1 year. The amphetamine study may be extended to include catecholamine measurements in patients pretreated with propranolol or haloperidol prior to the amphetamine infusion. We will continue to foster collaborative studies in which our contribution is the measurement of plasma catecholamines using liquid chromatography with electrochemical detection.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01933-01 HE

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Impedance Cardiography in Hypertension

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

PI:	David Goldstein, M.D., Ph.D.	Clinical Associate	HE	NHLBI
OTHER:	Harry R. Keiser, M.D.	Deputy Chief	HE	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.10	.10	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

We are determining the validity of impendance cardiography as a noninvasive measure of cardiac output and stroke volume in man, by comparison with results obtained using the Swan-Ganz thermodilution technique. If validated, the impedance cardiography technique offers the opportunity to measure cardiac output and peripheral resistance non-invasively in studying patients with essential hypertension.

PHS-6040
(Rev. 2-81)

Objectives: Impedance cardiography offers the unique advantage of determining cardiac output and stroke volume non-invasively on a beat-to-beat basis, potentially in ambulatory patients. The technique has not been validated to our satisfaction, though the equipment is commercially available. We therefore are attempting to validate the technique by comparison in the same patients with results obtained using the Swan-Ganz thermodilution technique.

Methods: Patients in the medical intensive care unit, or pre-operative patients, in whom invasive hemodynamic monitoring with a Swan-Ganz catheter is clinically indicated, will undergo simultaneous measurement using impedance cardiography and Swan-Ganz thermodilution techniques of cardiac output and stroke volume.

Results and their significance: Because this protocol is newly approved, only two patients have been tested so far. In one patient, who was undergoing hyperthermia as part of a cancer experimental therapeutic protocol, excellent agreement was obtained between the cardiac output measurements obtained using the two techniques (r = 0.89). In the other patient, results using impedance cardiography were similar to those obtained using the Fick technique (CO = 8.9 vs 9.6 L/min), when the thermodilution curves were unsatisfactory.

Proposed course of protocol: We will evaluate 10 patients using the two techniques. If the agreement across subjects is good, then we will introduce manipulations designed to change peripheral resistance or cardiac output to determine the agreement between the techniques within subjects. Once validated, the technique will be used in studying cardiac output and peripheral resistance in patients with hypertension. These studies should take 1 to 2 years.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01934-01 HE

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

The Sympathetic Nervous System and Hypertension

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

PI:	David Goldstein, M.D., Ph.D.	Clinical Associate	HE NHLBI
OTHER:	Harry R. Keiser, M.D.	Deputy Chief	HE NHLBI

COOPERATING UNITS (if any)

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Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

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TOTAL MANYEARS: .30	PROFESSIONAL: .30	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)

Using assay techniques developed and validated in this laboratory, we are measuring plasma norepinephrine as an indicator of sympathetic neural activity and plasma epinephrine as an indicator of sympathoadrenomedullary activity in hypertension.

PHS-6040
(Rev. 2-81)

Objectives: The results of recent literature reviews (1-4) suggest that venous plasma norepinephrine or epinephrine may be abnormally high in a proportion of patients with essential hypertension, suggesting in turn a pathogenetic role for accentuated sympathetic neural or sympathoadrenomedullary activity. We have developed and validated assay techniques for measuring plasma norepinephrine and epinephrine (5-7), and we can now use these techniques for testing hypotheses about the role of the sympathetic nervous system in hypertension.

By measuring arm arterial and venous catecholamines, we can assess the contributions of peripheral vascular sympathetic activity as reflected by arteriovenous differences in norepinephrine, as well as presumed uptake of epinephrine by peripheral vessels as reflected by arteriovenous differences in epinephrine.

Because the concentration of norepinephrine in the synaptic cleft is the complex product of neuronal reuptake, metabolic degradation, nonspecific uptake, and release from pre-synaptic storage granules, knowledge about these specific components is crucial for understanding sympathetic nervous system function in essential hypertension. Using L- and D-norepinephrine, only the former of which is taken up into storage granules and recognized by MAO, stereoselective components of norepinephrine disappearance can be quantitated. Similarly, since both D- and L-norepinephrine are taken up by nerve terminals, while isoproterenol is not, the difference in disappearance kinetics between norepinephrine and isoproterenol should provide a measure of specific norepinephrine uptake.

Instrumental cardiovascular conditioning can produce large magnitude elevations in blood pressure in baboons. This preparation provides a unique model for study of chronically repeated pressor episodes where the extent of pressure elevation is controllable. We are determining the role of sympathetic activity in mediating the blood pressure changes.

Adie's syndrome is a neurologic abnormality of unknown cause, the signs of which are characteristic pupillary abnormalities, depressed deep tendon reflexes, and often orthostatic hypotension. We had the opportunity to study mechanisms of neural circulatory control in such a patient with this syndrome, who also suffered severe hypertension of unknown cause.

Pheochromocytomas are catecholamine-secreting tumors which can cause hypertension. They represent the unusual situation of surgically curable hypertension. The diagnosis of pheochromocytoma has depended on urinary catecholamine metabolite measurement, arteriography, computed tomography, and exploratory laparotomy. We are determining the diagnostic usefulness of measuring blood levels of the direct products of pheochromocytoma secretion: plasma catecholamines.

Methods: For the study of arteriovenous differences in catecholamines, healthy persons and patients with essential hypertension undergo percutaneous insertion of plastic catheters into an antecubital vein and brachial artery. After 20 minutes of lying supine, they undergo 10 cc phlebotomies, and the blood is collected into chilled, heparinized, evacuated tubes. Arterial and venous plasma catecholamine concentrations are determined using liquid chromatography with electrochemical detection.

For the study of norepinephrine kinetics, healthy persons and patients with essential hypertension undergo insertion of antecubital catheters or needles in each arm. C-14 labelled D-norepinephrine, tritiated L-norepinephrine, and tritiated isoproterenol are infused together for 20 minutes, and repeated

blood samples taken during the infusion and for 20 minutes afterwards. The norepinephrine and isoproterenol peaks are separated using liquid chromatography with electrochemical detection and the C-14 and tritium counted using liquid scintillation spectrometry. The ratio of C-14 to tritium in norepinephrine provides a measure of the stereoselective components of norepinephrine disappearance, while the ratio of tritium in isoproterenol to that in norepinephrine provides a measure of specific norepinephrine uptake.

For the study of plasma catecholamines during instrumental cardiovascular conditioning in baboons, baboons are reinforced for increases in diastolic blood pressure during daily, 12-hour training sessions, where the criterion pressure is increased over the course of several weeks using a shaping procedure. Plasma catecholamines are measured using liquid chromatography with electrochemical detection on blood samples drawn from chronically indwelling arterial catheters before and during the conditioning sessions.

For the study of neural circulatory control in Adie's syndrome, we measured plasma catecholamines during supine rest, during standing, and after insulin-induced hypoglycemia; arteriovenous differences in plasma catecholamines; plasma disappearance kinetics of injected radiolabelled D- and L-norepinephrine; and measured baroreflex sensitivity using 8 different techniques.

Results and their significance: In the study of arteriovenous catecholamine differences, 4 normotensives and 9 patients with essential hypertension have been studied. Hypertensives have shown higher arterial and venous norepinephrine levels (314 vs. 145 pg/ml and 345 vs. 153 pg/ml) than normotensives, as well as greater arteriovenous differences in norepinephrine (31 vs. 8 pg/ml), although too few data points have been collected to allow statistical testing. Hypertensives have also shown higher arterial and venous epinephrine levels (125 vs. 84 pg/ml and 96 vs. 63 pg/ml), but no accentuation of arteriovenous differences in epinephrine.

These results suggest that accentuated peripheral vascular sympathetic activity occurs in essential hypertension, and that both abnormal sympathetic neural and sympathoadrenomedullary activity characterize a proportion of patients with essential hypertension.

In the study of norepinephrine kinetics, one hypertensive and 6 normotensive patients have undergone infusions. The ratio of isoproterenol to norepinephrine has increased with time after the end of the infusion, consistent with reuptake of norepinephrine but not isoproterenol. The ratio of D- to L-norepinephrine also has increased with time, consistent with uptake of L- but not D-norepinephrine into storage granules. The above-described procedure therefore does allow quantitation of stereoselective and reuptake components of norepinephrine disappearance kinetics.

In the study of instrumental diastolic blood pressure conditioning in baboons (8), pre-training baseline norepinephrine averaged 364 pg/ml, epinephrine 253 pg/ml, dopamine 270 pg/ml, and renin activity 2.6 ng/ml/hr. During the training sessions, systolic pressure increased significantly 19 mm Hg (15%), diastolic pressure 20 mm Hg (30%), and heart rate 33 bpm (33%) compared with pre-session values. The blood pressure elevations were completely reversible, demonstrating that chronically repeated pressor episodes in otherwise healthy individuals do not themselves

cause sustained hypertension. During the sessions, norepinephrine increased significantly by 253 pg/ml (48%), and epinephrine 130 pg/ml, while dopamine and renin activity were unchanged. Plasma norepinephrine correlated positively with systolic and diastolic pressures in all baboons tested, in both baseline and training conditions. To the extent that plasma norepinephrine reflects sympathetic neural activity, these findings demonstrate an association between sympathetic activity and blood pressure in this hypertensive model.

In the study of the patient with Adie's syndrome, resting norepinephrine levels were normal, and the patient showed a normal increment in norepinephrine with standing. Baroreflex sensitivity was markedly depressed, by all techniques in which R-R interval was a dependent measure, yet heart rate responses to isoproterenol and atropine were essentially normal. Data derived from the norepinephrine infusion have not been analyzed yet. The results suggest an abnormality of central integration of reflex circulatory control in this syndrome.

Across 12 patients with surgery-proven pheochromocytoma and 13 with hypertension but without pheochromocytoma, the plasma norepinephrine distributions showed only slight overlap. Two patients with pheochromocytoma showed plasma norepinephrine levels within two standard deviations of the mean of the non-pheochromocytoma patients; the remainder showed plasma norepinephrine levels exceeding 2 SD's above the non-pheochromocytoma mean. These results suggest that exceedingly high norepinephrine levels--above 600 pg/ml--in resting, supine patients with hypertension support the diagnosis of pheochromocytoma, but that levels less than about 600 pg/ml do not exclude the diagnosis.

Proposed course of project: The study of arteriovenous catecholamine differences will be continued until about 15 normotensives and 25 hypertensives have been included. The study of norepinephrine kinetics will be continued until about 15 patients with hypertension and 15 controls have been tested. These studies should be complete in 1 to 2 years.

Because the conditioning procedure in baboons has not produced sustained hypertension when the sessions have ended, we wish to determine whether salt loading interacts with the conditioning to produce sustained hypertension. We also will repeat catecholamine measurements in baboons trained to decrease diastolic pressure. These studies should take 2 to 3 years.

Plasma catecholamine determinations in patients in whom the diagnosis of pheochromocytoma is entertained will be conducted as an ongoing clinical service.

In general, we anticipate that our studies of plasma catecholamines will be expanded to other cardiovascular diseases, since the sympathetic nervous system has been invoked to explain a wide variety of pathophysiologic phenomena in clinical cardiology (9). We hope to conduct such studies in collaboration with the Cardiology Branch in the future.

Publications:

1. Kopin I.J., Goldstein, D.S., Feuerstein, G.Z.: The sympathetic nervous system and hypertension. Frontiers in Hypertension Research. (In press).

2. Goldstein, D.S.: Plasma norepinephrine in essential hypertension: A study of the studies. Hypertension 3:48, 1981.

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5. Goldstein, D.S., Feuerstein, G.Z., Izzo, J.L., Jr., Kopin, I.J., Keiser, H.R.: Validity and reliability of liquid chromatography with electrochemical detection for measuring plasma levels of norepinephrine and epinephrine in man. Life Sciences 28:467, 1981.

6. Goldstein, D.S., Feuerstein, G.Z.: Improved reliability of the liquid chromatography-electrochemical detection assay technique for measuring plasma epinephrine. Clin. Chem. 27:508, 1981.

7. Shoup, R.E., Kissinger, P.T., Goldstein, D.S.: Rapid liquid chromatographic methods for assay of norepinephrine, epinephrine, and dopamine in biological fluids and tissues. Norepinephrine: Clinical Aspects. (In press).

8. Goldstein, D.S., Harris, A.H., Izzo, J.L., Jr., Turkkan, J.S., Keiser, H.R.: Plasma catecholamines and renin during operant blood pressure conditioning in baboons. Physiol. Behavior 26:33, 1981.

9. Goldstein, D.S.: Plasma norepinephrine as an indicator of sympathetic neural activity in clinical cardiology. American Journal of Cardiology. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01935-01 HE

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Studies of Resistance Vessels 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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COOPERATING UNITS (if any)

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Experimental Therapeutics

INSTITUTE AND LOCATION

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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)

Blood flow in the hands of hypertensive and normotensive subjects was determined by venous occlusion plethysmography during maximal vasodilatation and under different levels of transmural pressure. Hypertensive subjects had significantly reduced blood flow in the hands during maximal vasodilatation.

PHS-6040
(Rev. 2-81)

Objectives: Patients with established essential hypertension show increased peripheral vascular resistance with a normal cardiac output. The present studies were initiated in an attempt to determine the extent to which structural changes in resistance vessels were present which might contribute to the increased vascular resistance of hypertensive patients. Reduced ability of hypertensive patients to maximally dilate the vessels of the hand was considered to provide evidence for structural changes. A new technique was utilized wherein varying levels of external pressure were imposed on the hand to produce different levels of transmural pressure. This permitted comparison of hypertensives and normotensives at the same level of transmural pressure.

Methods: Blood flow in the hand during maximum vasodilatation was determined using venous occlusion plethysmography. Blood pressure was measured by the arm-cuff method. Maximum vasodilatation was induced by maintaining a temperature of 43°C in the water-filled plethysmograph and by rendering the hand ischemic for periods of ten minutes. The pressure in the plethysmograph was varied in different runs by raising a column of fluid to different levels above the plethysmograph. When the different levels of transmural pressure achieved by varying the plethysmograph pressure were plotted against flow a straight line was obtained. A least mean squares regression line was calculated for each subject and the flow at a mean transmural pressure of 85 mm of Hg was computed. Because of carryover of vasodilating effects in successive runs maximum effects were not achieved until the second or third run; the first two runs were, therefore, arbitrarily discarded in all computations.

Subjects: The subjects were seven normal volunteers and seven patients with untreated, uncomplicated mild to moderate essential hypertension. The hypertensives were from 28 to 50 years of age and the normotensives from 20 to 50 years of age; the average age of the hypertensives was 40 and that of the normotensives was 35.

Major Findings: Mean blood flow at a transmural pressure of 85 mm Hg was significantly greater in normotensive than in hypertensive subjects (43 vs 31 ml/min/100 ml of tissue). Only one of seven hypertensive subjects overlapped with the normotensives.

Significance to Biomedical Research and Institute Programs: Changes in vascular structure are a fundamental mechanism that has been proposed to explain the initiation and perpetuation of essential hypertension. The present study, utilizing a new technique, presents evidence that resistance vessels of hypertensives differ from those of hypertensives. The new technique will facilitate studies of vessels in various stages of hypertension and will permit correlations with ongoing studies of sympathetic nervous system function and baroreceptor controls.

Proposed Course of Project: Studies will be pursued in patients with early stages of hypertension and in patients whose blood pressure has been controlled by drugs.

Publications:

1. Izzo, J.L., Horwitz, D., and Keiser, H.R.: Physiologic mechanisms opposing the hemodynamic effects of prazosin. Clin. Pharmacol. Therap. 29: 7-11, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01936-01 HE

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Effect of Aldosterone and Vasopressin on Kallikrein-Like Activity in the Toad Bladder

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

PI:	Arthur B. Pitterman, M.D.	Senior Staff Fellow	HE NHLBI
OTHER:	Joseph S. Handler, M.D.	Section Head	KE NHLBI
	Harry R. Keiser, M.D.	Deputy Chief	HE NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.50	.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)

The kallikrein-like enzyme activity of the toad bladder was measured after 24 hour incubations with either aldosterone 10^{-7}, vasopressin 10^{-7}, or Ringer's solution. There was no significant change in kallikrein-like activity after aldosterone or vasopressin incubation when compared with the control.

PHS-6040
(Rev. 2-81)

Objectives: Margolius discovered a kallikrein-like enzyme in the urinary bladder and skin of Bufo marinus toads. This kallikrein-like enzyme is strongly inhibited by amiloride and only slightly inhibited by triamterene (J. Clin. Invest. 65: 1343, 1980). This same pattern of inhibition has also been demonstrated with purified rat and human urinary kallikrein in vitro.

Our objective was to investigate if aldosterone or vasopressin could increase the kallikrein-like activity of the toad bladder, thus exploring the possibility that kallikrein is controlled by circulating aldosterone.

Methods: Bufo marinus toads were housed in plastic cages and kept on moist pads at room temperature. The urinary bladders were removed from doubly pithed toads. The hemibladders were rinsed, and suspended in a glass incubation chamber. Three ml of Ringer's solution containing 1 mg/ml each of penicillin and streptomycin was placed on the epithelial side of the bladder sacks while the serosa side was bathed in a similar solution which also contained 20 mM glucose. Either aldosterone 10^{-7}M or vasopressin 10^{-7} were randomly added to the serosal side of one hemibladder from each pair of bladders and incubated for 24 hours. Then the bladder's epithelial solution and epithelial cells were collected and frozen in liquid nitrogen.

The epithelial cells were homogenized, incubated with 0.5% deoxycholate for 30 minutes and the mixture centrifuged. The supinate was desalted by gel filtration through Sephadex G-25 and then acid treated for 30 min (pH 2.5). The kallikrein-like activity of the serosal solution and cell extract were determined, via the radiochemical Tosyly Arg - OMe esterase assay developed by Dr. John Pisano's laboratory, and the spectrophotometric assay employing S - 2266 chromogenic substrate from Kabi Diagnostica Stockholm, Sweden.

Results: The epithelial cell extract and the serosal solution contained a large amount of nonspecific protease activity which is not inhibited by trasylol (10 KIU/ml). Trasylol (pancreatic trypsin inhibitor) is a potent inhibitor of both glandular and plasma kallikrein. There was no significant difference between the kallikrein activity of the treated bladders and the control bladders. We could not demonstrate that aldosterone or vasopressin had any effect on the toad bladder kallikrein-like activity. With a better purification of the toad bladder kallikrein-like enzyme it may be possible to demonstrate that aldosterone will cause a difference in the toad bladder kallikrein-like activity. Our technics are probably not sensitive enough to detect changes in the true kallikrein-like enzyme in the presence of a relatively large amount of nonspecific proteases. Margolius was apparently able to remove the interference caused by the nonspecific proteases. For this reason, Margolius was able to report that trasylol inhibited the toad bladder kallikrein-like enzyme activity while we could not.

Proposed Course of Project: We have decided not to pursue this project any further due to the insensitiveness of our ability to measure the toad bladder kallikrein-like enzyme.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01937-01 HE

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Modification of Endotoxic Shock by Vagotomy

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

PI:	Arthur B. Pitterman, M.D.	Sr. Staff Fellow	HE	NHLBI
	Daniel J. Goldstein, M.D.	Expert	HE	NHLBI
OTHER:	Harry R. Keiser, M.D.	Deputy Chief	HE	NHLBI
	Gerald Kelly	Biol. Lab. Tech.	HE	NHLBI

COOPERATING UNITS (if any)

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Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.50	.38	.12

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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 vagus nerve in the hypotension and death induced by lipopolysaccharide is being assessed in awake rats. Our preliminary data indicate that abdominal vagotomy modifies the circulatory response to endotoxic shock and improves survival while treatment with methylatropine nitrate does not.

PHS-6040
(Rev. 2-81)

Objectives: Naloxone reversal of lipopolysaccharide induced hypotension suggests that endorphins are involved in this type of shock (Nature 257: 450, 1978). Opiate antagonists not only improve the hemodynamics but also the survival of rats after spinal injury and hypovolemic shock (Science 211: 493, 1980; Science 205: 317, 1979). Bilateral vagotomy but not methylatropine nitrate abolished the decrease in blood pressure in rats following morphine injection (Brit. J. Pharmacol. 7: 542, 1952).

Our objective was to investigate the effect of bilateral vagotomy on the blood pressure response and survival after lipopolysaccharide administration.

Methods: Male Sprague-Dawley rats had abdominal vagotomy (bilaterally) and similar rats had sham operations. Three weeks post surgery indwelling venous and arterial catheters (PE 50) were inserted into the left jugular vein and tail artery. The rats were placed in individual cages in which they were free to move about with their catheters exiting from the back of their necks (Am. J. Physiol. 243: H690, 1978; J. Pharmacol. Exper. Therap. 212: 441, 1980). Catheters were kept patent with 0.5 ml of 500 units/ml sodium heparin in normal saline. Approximately 24 hours after implantation of the catheters, the arterial catheter was connected to a pressure transducer and recorder. Lipopolysaccharide 70 mg/kg was slowly administered via the venous catheter. The blood pressure was recorded for 2 hours after the lipopolysaccharide injection and the animal was observed 24 hours later for survival.

Awake and freely moving rats were used in the study to eliminate the possible changes in vascular, hormonal, neurotransmitter and endorphin effects that may occur with restrained or anesthetized animals.

Results: Preliminary data from 4 vagotomized and 4 control animals show a reversal of the hypotension which occurs approximately 45 minutes after the lipopolysaccharide administration. The diastolic blood pressure in three of the 4 vagotomized rats did not fall below the blood pressure prior to receiving the endotoxin. The diastolic blood pressure of the fourth vagotomized rat decreased 14% below its basal level. Three of the four control rats died within one hour after endotoxin administration. The fourth control rat's diastolic blood pressure decreased 2% from its basal level. In another preliminary study 10 vagotomized and 10 sham rats received lipopolysaccharide 70 mg/kg via the tail vein and all the animals except 4 vagotomized rats died within 24 hours. Methylatropine nitrate did not show any improvement in hemodynamic parameters or survival. These data suggest that the ascending nervous pathway of the vagus may be involved in the hypotensive response during shock. Since naloxone reverses endotoxic shock, it is possible that the vagus releases endorphins which cause a decrease in the blood pressure.

Proposed Course of Project: We plan to increase the sample size of this study to be able to statistically determine if vagotomy affects endotoxic shock. If abdominal vagotomy reverses endotoxic shock, we will investigate the following:

1) The effect of acute vagotomy on endotoxic shock.

2) The effect of selective abdominal vagotomy (cutting the branches which modify the portal system) on endotoxic shock.

3) The effect of vagotomy in other shock models, for example, acute hemorrhagic shock.

4) The difference in cardiac output and arterial blood pressure between vagotomized and sham animals during endotoxic shock.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01938-01 HE

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Role of Vagal Afferents in Circulatory Control

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

PI:	Daniel J. Goldstein, M.D.	Expert	HE	NHLBI
	Ingeborg Hanbauer, Ph.D.	Pharmacologist	HE	NHLBI
OTHER:	Mr. Gerald Kelly	Biol. Lab. Tech.	HE	NHLBI
	Harry R. Keiser, M.D.	Deputy Chief	HE	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.20	.15	.05

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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 shown that several opioid peptides exert their hypotensive effects in the anesthesized rat via the activation of vagal afferent fibers.

PHS-6040
(Rev. 2-81)

Objectives: The blood-brain barrier is essentially impermeable to peptides, but many of the brain-gut peptides exert significant central effects when administered peripherally. We and others have shown that bilateral subdiaphragmatic vagotomy abolishes the dipsogenic effect of i.v. perfused angiotensin II. Smith et al. have demonstrated that cholecystokinin s.c. induces satiety in fasted animals and that this effect is dependent on the integrity of the abdominal vagal trunks. The hypotensive effect of morphine is also abolished by vagotomy. In these three examples, the administration of atropine does not mimic the effects of surgical vagotomy, leading to the inference that these molecules trigger peripheral receptors which in turn activate afferent vagal pathways which convey information to the central nervous system. Therefore, our objective is to determine whether the brain-gut peptides exert their vascular effects by activating afferent vagal fibers.

Methods: Male Sprague-Dawley rats, weighing 300 grams each, are anesthetized with urethane. Venous and arterial catheters are implanted and blood pressure and heart rate monitored continuously.

Results and their significance: So far, we have found that the hypotensive response to met-enkephalin (200 μg/kg), D-ala-met-enkephalin (100 μg/kg), and β-endorphin (500 μg/kg) is considerably attenuated or completely abolished by subdiaphragmatic bilateral vagotomy. Atropine methyl-sulphate (10 mg/kg) does not mimic the effect of surgical vagotomy and has no effect on the hypotensive response to these peptides. Our results indicate that peripheral receptors and vagal afferent fibers might be extremely important in the regulation of blood pressure.

Proposed course of study: We will continue the study of the cardiovascular effects of other brain-gut peptides and will give special attention to the large molecular weight polypeptides of adrenal origin with opioid sequences.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01939-01 HE

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Neuronal Specific Enolase in Pheochromocytomas

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

PI:	Daniel J. Goldstein, M.D.	Expert	HE	NHLBI
OTHERS:	Ilona Linnoila, M.D.	Research Associate	DCBD	NCI
	Lee Eiden, Ph.D.	Research Associate	LCS	NIMH
	Michael Brownstein, M.D., Ph.D.	Pharmacologist	LCS	NIMH
	Harry R. Keiser, M.D.	Deputy Chief	HE	NHLBI

COOPERATING UNITS (if any)

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Hypertension-Endocrine Branch

SECTION
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INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.60	.60	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Pheochromocytomas contain considerable amounts of neuronal specific enolase (NSE), a biochemical marker for peptidergic neurons and APUD cells. However, the plasma concentration of NSE in pheochromocytoma patients is no higher than that found in normal individuals.

PHS-6040
(Rev. 2-81)

Objectives: Dr. Paul Marangos has shown that an isozyme of the glycolytic enzyme enolase, neuronal specific enolase (NSE), is uniquely present in peptidergic neurons and APUD cells. As the adrenal medulla is especially rich in NSE, it was of certain interest to know if human pheochromocytomas were also NSE positive and to explore its potential usefulness as a biochemical marker of this tumor. We started a collaborative project to answer the following questions:

1) Does neoplasic adrenomedullary tissue contain NSE?

2) Are plasma concentrations of NSE in pheochromocytoma patients higher than those of normotensive and other types of hypertensive patients?

3) Could the estimation of NSE plasma concentrations be of diagnostic and/or prognostic value in the evaluation of pheochromocytoma patients?

Methods: NSE was measured by radioimmunoassay and by immunocytochemistry by the techniques developed (and published) by Dr. Paul Marangos. Eleven pheochromocytoma patients were studied, and 12 controls, matched by age, race, and sex. Plasma and tissue sections were analyzed in all of them. Two tumors and 2 normal adrenal medullas were assayed biochemically.

Results: We found that (a) the pheochromocytomas are extraordinarily rich in NSE, as evidenced by biochemical analysis and immunohistochemistry; (b) plasma levels of NSF are not consistently elevated in pheochromocytoma patients (3-17 ng/ml, vs 2.5-10 ng/ml in controls, with 8 pheochromocytoma patients in the normal range); (c) surgical removal of the tumor does not modify the plasma NSE concentration; (d) the amount of tumor tissue was not proportional to the plasma concentration of NSE.

These data show:

1) pheochromocytoma cells are differentiated APUD cells (undifferentiated cells of neural origin are NSE negative).

2) the biology of the pheochromocytoma is different from the oat cell carcinoma, another tumor with high NSE content which is accompanied by very high NSE plasma concentration.

3) the enzyme NSE is not co-secreted with norepinephrine by pheochromocytomas.

On the other hand, the high concentrations of NSE in tumor tissue strongly suggest that the neoplastic cells contain peptides of the brain-gut class. We do not find increased concentrations of met-enkephalin immunoreactive material before and after proteolytic treatment of the plasma of pheochromocytoma patients. Met-enkephalin immunoreactivity in plasma is not altered after the removal of the tumor. Confirming this biochemical information, we do not find an increased number of met-enkephalin-positive cells in the tumors.

An additional finding of these studies has been the surprisingly high NSE plasma concentrations recorded in 17 essential hypertensive patients (7-36 ng/ml, with only 3 patients in the normal range). We do not know yet if these values are

significantly different from those of normotensive patients as our control values are not matched for age, sex, or race.

Proposed course of study: I plan to continue the investigation of NSE plasma concentrations in essential hypertensive patients and to study a possible correlation with norepinephrine values. Along the original line of this project, I am trying to identify (by bioassay) the peptide(s) contained in pheochromocytoma tissue. A collaboration will be initiated with Dr. Sidney Udenfriend (Roche Institute for Molecular Biology) to analyze the large molecular weight opioid peptides in pheochromocytoma tissue and in the plasma of pheochromocytoma patients.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01940-01 HE

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Naloxone Reversal of Hageman Factor-Induced Hypotension in the Rat

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

PI:	Daniel J. Goldstein, M.D.	Expert	HE	NHLBI
	Arthur B. Pitterman, M.D.	Sr. Staff Fellow	HE	NHLBI
OTHER:	Erminio Costa, M.D.	Chief, Lab. of Preclinical Pharmacology		NIMH
	Marian Warner, B.S.	Biologist	HE	NHLBI
	Gerald Kelly	Biol. Lab. Tech.	HE	NHLBI
	Harry R. Keiser, M.D.	Deputy Chief	HE	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.20	.15	.05

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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 hypotensive effect induced by the intravenous administration of a purified activated Hageman Factor fragment in the rat is attenuated by naloxone.

PHS-6040
(Rev. 2-81)

Objectives: The classical picture of enkephalin biosynthesis has proven to be incomplete or wrong. Many large molecular weight opioids are made by the adrenal medulla and their physiological function and biochemical modifications, once they are released into the blood stream, are essentially unknown.

Hageman Factor plays a central role in the activation of many cascades of proteolytic activity in plasma. The objective of this study was to determine if this enzyme could be involved in the generation of active hypotensive opioid peptides from circulating precursors.

Methods: Male Sprague-Dawley rats (300 grams) were anesthetized with pentobarbital, implanted with venous and arterial catheters, and their blood pressure was recorded. The purified, activated Hageman Factor fragment was the generous gift of Dr. J. J. Pisano.

Results and their significance: We have found that the response to a second injection of Hageman Factor (500 ng/kg) is significantly attenuated by pretreatment with naloxone (4 mg/kg). It is impossible to estimate the effect of naloxone after a first Hageman Factor injection because of the great variability in response from rat to rat. This led us to compare the following injection sequences: HF - Saline - HF, to HF - Naloxone - HF, expressing the results of the percent of hypotension induced by the second Hageman Factor injection with respect to the first. The effect of naloxone is specific, because this drug does not block the hypotensive response to bradykinin, urinary kallikrein or histamine.

Proposed Course of Study: It is necessary to obtain biochemical confirmation of these pharmacological results. Initial experiments done in collaboration with Dr. Erminio Costa have been inconclusive because of the small amounts detected by the met-enkephalin radioimmunoassay of rat plasma. We plan to explore the generation of opioid peptides of human plasma activated in vitro by dextran sulphate.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 HL 01941-01 HE

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Role of Prostaglandins in Primary Aldosteronism

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

PI: John R. Gill, Jr., M.D. Senior Investigator HE NHLBI

COOPERATING UNITS (if any)

Dr. John A. Oates, Division of Clinical Pharmacology, Vanderbilt University School of Medicine, Nashville, Tennessee

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	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)

Hyporeninemia, aldosteronism and hypertension characterize primary aldosteronism due to an aldosterone-producing adrenal adenoma. Patients with this disorder may have an overproduction of prostaglandin E_2 by the kidneys that is probably the result of potassium depletion. Inhibition of prostaglandin synthesis did not affect production of aldosterone by the adenoma or production of 17-hydroxycorticosteroids by the adrenal glands. Also, prostaglandin synthetase inhibition did not decrease creatinine clearance or sodium and potassium excretion as it has in other disorders with increased renal synthesis of prostaglandin E_2. The results suggest that prostaglandins are not necessary for adrenal steroid biogenesis and that they may not have a role in the hypertension of primary aldosteronism.

PHS-6040
(Rev. 2-81)

Project Description and Objective:

In vitro studies indicate that prostaglandins stimulate the production of aldosterone and cortisol by adrenal tissue. Also, patients with potassium depletion and an overproduction of prostaglandins of the E and F series may also overproduce aldosterone. Treatment of these patients with an inhibitor of prostaglandin synthesis corrects both the overproduction of prostaglandins and aldosterone and suggests that the overproduction of prostaglandins is the basis for the increased secretion of aldosterone. This effect of aldosterone synthetase inhibition on aldosterone biogenesis may result directly from an inhibition of renin release, or both. In addition to the findings that prostaglandins may mediate an increase in aldosterone production, other studies indicate that treatment of rats and dogs with desoxycorticosterone increases urinary prostaglandin E_2. Therefore, patients with primary aldosteronism were studied to determine the effects of aldosterone on urinary prostaglandin E_2 and to assess the role of prostaglandins in adrenal steroidogenesis.

Methods Employed:

Seven patients (four men and three women) with an aldosterone-producing adenoma and primary aldosteronism were given constant diets which contained 109 mEq/day of sodium. After four days of control observations, the patients were treated with indomethacin, 150 mg/day for eight days. Urine was collected daily for determination of aldosterone, 17-hydroxycorticosteroids, creatinine, sodium and potassium. Also, aliquots of urine were taken during the control and treatment periods for the measurement of prostaglandin E_2 by gas chromatography and mass spectrometry. Blood was drawn every two days for the determination of potassium, creatinine and plasma renin activity.

Major Findings and Significance:

The control observations and the results of treatment with indomethacin are shown in the table below:

Regimen	Serum K^+ mEq/L	Clearance Creatinine ml/min	Plasma Renin Activity ng/min/hr	Urine Na^+ mEq/d	Urine K^+ mEq/d	Urine Aldosterone μg/d	Urine 17-OH Corticosteroids ng/d	Urine PGE_2 ng/d	Blood Pressure mmHg
Control	2.8	81	0.12	91	79	17.9	10.1	618	174/102
Indomethacin	2.8	83	0.18	96	78	16.6	10.8	82	178/103
P						>0.2	>0.3	<0.01	

Mean urinary PGE_2 for all the patients was 618 ng/day and the mean value for the three women in the study was 410 ng/day (normal value for women 185 ± 81 ng/day). (It is difficult to evaluate urinary PGE_2 in men because of variable contributions by prostate and seminal vesicles). In previous studies, treatment of dogs with desoxycorticosterone was associated with an increase in urinary PGE_2 and this suggests that the supranormal values for PGE_2 in the women in the present study resulted from the overproduction of aldosterone. As the increase in urinary PGE_2 that accompanied desoxycorticosterone in the dogs could be prevented or corrected by prevention or correction of hypokalemia, presumably the high urinary PGE_2 in patients with primary aldosteronism was the result of the associated potassium depletion.

Treatment of the patients with indomethacin decreased urinary prostaglandin E_2 from 618 to 82 ng/d. The inhibition of PGE_2 synthesis did not affect production of aldosterone by the adenoma of production of 17-hydroxy-corticosterones by the adrenal glands. Plasma renin activity was suppressed and did not change with indomethacin. These results suggest that although prostaglandins may stimulate steroidogenesis _in vitro_, they are not essential for steroidogenesis _in vivo_. The results also suggest that the decrease in aldosterone with indomethacin in Bartter's syndrome was probably secondary to the decrease in plasma renin activity rather than the result of an inhibition of adrenal prostaglandin synthesis.

Treatment with indomethacin did not decrease creatinine clearance or sodium and potassium excretion in the patients with primary aldosteronism as it has in other disorders with increased renal synthesis of prostaglandin E_2 such as Bartter's syndrome, hepatic cirrhosis and lupus erythematosis. The patients with primary aldosteronism had suppressed plasma renin activity and hypertension rather than normal or high plasma renin activity and normal blood pressure that characterized the patients with the other disorders. These differences in plasma renin activity and blood pressure probably account for the differences in the renal response to indomethacin.

Blood pressure in these patients with primary aldosteronism was unaffected by treatment with indomethacin and this suggests that prostaglandins probably have no role in this form of hypertension.

Proposed Course of Project:

Patients suspected of having primary aldosteronism will continue to be evaluated for the disorder. We will also continue to measure urinary prostaglandin E_2 in the women with primary aldosteronism to determine the extent of the occurrence of high urinary prostaglandin E_2.

Publications:

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2. Güllner, H.-G., Tiwari, J.L., Terasaki, P.I., Gill, J.R., Jr. and Bartter, F.C.: Genetic linkage between histocompatibility antigens (HLA) and a new syndrome of familial hypokalemia. IRCS Medical Science 8: 369-370, 1980.

3. Güllner, H.-G., Smith, J.B., Cerletti, C., Gill, J.R., Jr. and Bartter, F.C.: Correction of increased prostacyclin synthesis in Bartter's syndrome by indomethacin treatment. Prostaglandins and Medicine 4: 65-72, 1980.

4. Güllner, H.-G., Gill, J.R., Jr., Bartter, F.C. and Düsing, R.: The role of the prostaglandin system in the regulation of renal function in normal women. Amer. J. Med. 69: 718-724, 1980.

5. Düsing, R., Bartter, F.C., Gill, J.R., Jr., Güllner, H.-G. and Lake, C.R., Jr.: Effects of moderate short-term potassium depletion in normal humans: The role of prostaglandins. Prostaglandins 20: 971-979, 1980.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01942-01 HE

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Adrenergic Nervous System Function in Experimentally-induced Potassium Depletion

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

PI:	Hans-Georg Güllner, M.D.	Guest Worker	HE NHLBI
Other:	John R. Gill, Jr., M.D.	Senior Investigator	HE NHLBI

COOPERATING UNITS (if any)

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LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	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)

It has previously been shown that patients with Bartter's syndrome have an increase in sympatho-adrenal function as indicated by a significant increase in urinary norepinephrine and epinephrine excretion. Correction of the overproduction of prostaglandins by treatment with an inhibitor of prostaglandin synthesis restores urinary norepinephrine and epinephrine to normal. To test the hypothesis that potassium depletion may be the basis for the increase in prostaglandins and in sympathetic nervous system activity, hypokalemia was experimentally induced in rats. The potassium-depleted rats showed an increase in plasma norepinephrine, epinephrine and plasma renin activity. The results indicate that potassium depletion is a potent stimulus for the sympathetic nervous system and the renin-angiotensin system.

PHS-6040
(Rev. 2-81)

Project Description and Objectives:

Patients with Bartter's syndrome are resistant to the pressor effects of norepinephrine and show increased urinary excretion of epinephrine and norepinephrine. The pressor resistance and increase in catecholamine excretion can be corrected by treatment with inhibitors of prostaglandin synthesis that correct the overproduction of prostaglandins. It is not clear, however, whether the increase in sympathetic nervous activity is a specific feature of patients with Bartter's syndrome or whether it is secondary to the associated potassium depletion. The present studies were performed to evaluate the effect of potassium depletion on sympathetic nervous system function.

Methods Employed:

Two groups of Fischer rats were studied. One group (10 rats) was fed a synthetic control diet, a second group (10 rats) was given the same diet from which the potassium had been removed (potassium content 0.0025%, v/v) for 45 days. At the end of the experiment the rats were housed in individual metabolic cages and a 24-hour urine was collected from each rat. Blood was collected by puncture of the aorta under light pentobarbital anesthesia for measurement of plasma epinephrine, norepinephrine, plasma renin activity and serum sodium, potassium and chloride. Plasma epinephrine and norepinephrine were measured by a COMT-based radioenzymatic assay. Plasma renin activity was measured by radioimmunoassay of angiotensin I. For evaluation of the data, the control group was compared with the potassium-depleted group.

Major Findings and Significance:

The control observations and results of potassium depletion are shown in the table below:

	Control		Hypokalemia
Epinephrine (pg/ml)	393±61	$p<0.005$	1488±275
Norepinephrine (pg/ml)	303±34	$p<0.05$	552±101
Plasma Renin Activity (ng/ml/hr)	22.3±2.3	$p<0.001$	44.3±3.6
Plasma Aldosterone Concentration (ng/dl)	60.3±9.2	$p<0.01$	28.8±1.6
Potassium (mEq/L)	3.5±0.1	$p<0.001$	2.4±0.1
Magnesium (mEq/L)	1.64±0.04	$p<0.01$	1.89±0.04
Sodium (mEq/L)	148±1	$p<0.001$	151±1
Chloride (mEq/L)	95.0±0.3	$p<0.001$	84.1±1.0

The low potassium diet decreased serum potassium to 2.4 ± 0.1 mEq/L and increased serum magnesium to 1.89 ± 0.04 mEq/L but did not produce a significant change in body weight. Plasma renin activity increased from 22.3 ± 2.3 to 44.3 ± 3.6 ng/ml/hr and, despite this increase, plasma aldosterone concentration decreased from 60.3 ± 9.2 ng/dl to 28.8 ± 1.6 ng/dl. A paradoxical decrease in aldosterone concentration in association with an increase in plasma renin activity is a feature of severe potassium depletion and represents a direct effect of hypokalemia on aldosterone biogenesis. the increase in plasma epinephrine and norepinephrine indicate that potassium depletion can stimulate the sympathetic nervous system as well as the renin-angiotensin system. The increase in activity of these two pressor systems, probably occurs in response to the arterial vasodilatation that has been observed in potassium depletion. These findings are consistent with the current concept that potassium depletion may be the proximal cause for the increase in sympathetic nervous system function and hyperreninemia observed in Bartter's syndrome.

Proposed Course of Project:

Measurements of the urinary excretion of epinephrine, norepinephrine and dopamine are in process. Similarly, the urinary excretion of prostaglandin E_2 and of 6-keto-$PGF_{1\alpha}$ will be measured. In future experiments, the potassium-deficient animals will be treated with indomethacin, an inhibitor of prostaglandin synthesis, to examine whether the increase in adrenergic activity is dependent on prostaglandin overproduction. Prostaglandins and catecholamines in urine will be measured daily throughout the course of the study to determine the degree of hypokalemia necessary to produce changes in these hormones. Also, if both prostaglandin E_2 and 6-keto prostaglandin $F_{1\alpha}$ are increased, then urinary prostaglandins will be measured daily during the development of hypokalemia to determine if there is a dissociation in the overproduction of these two prostaglandins.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01943-01 HE

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Effect of Bradykinin on Renal Function in Dogs Treated With Indomethacin or Propranolol

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

PI:	John Yun, Ph.D.	Guest Worker	HE NHLBI
Other:	John R. Gill, Jr., M.D.	Senior Investigator	HE NHLBI
	Harry R. Keiser, M.D.	Deputy Chief	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The effect of bradykinin (BK) on renal function was examined in anesthetized dogs with or without treatment with either indomethacin or propranolol.

PHS-6040
(Rev. 2-81)

Project Description and Objectives:

Renal arterial infusion of bradykinin (BK) is known to produce an increase in salt and water excretion and in renal plasma flow (RPF). McGiff et al. reported that renal arterial infusion of BK caused an increase in the release of prostaglandin E (PGE)-like substance and suggested that the effect of BK on renal function may be mediated through prostaglandins. If this is true, administration of an inhibitor of prostaglandin synthesis should prevent rises in salt and water excretion and in RPF induced by BK. The purpose of this study was to determine whether indomethacin, an inhibitor of prostaglandin synthetase, would prevent increases in salt and water excretion and in RPF produced by renal arterial infusion of BK. Since it is also possible that BK produces diuresis and natriuresis by activating the beta adrenergic receptor, it was also the purpose of this study to determine whether beta-receptor blockade with propranolol would inhibit the diuretic and natriuretic response to BK.

Methods Employed:

Mongrel dogs were given a diet containing 180 mEq/d of sodium for one week before study. Renal clearances, with periods of 20 minutes, were performed according to the three protocols described below.

In the first series of experiments, after 3 control clearance periods, BK (3 μg/min) was infused into a renal artery for seven clearance periods followed by two post-control periods.

In the second series, after a control period, Indomethacin (5 mg/kg) was injected into the femoral venous catheter, followed by a sustaining infusion of 2.1 - 3.2 mg/kg/hr. After urine and blood samples had been collected for 2 clearance periods, BK (3 μg/min) was infused into the renal artery for seven clearance periods. This was followed by two post-control periods.

In the third series, the experimental procedures were the same as those for series 2 except that propranolol (5 mg/kg, followed by 2.8 - 3.4 mg/kg/hr) instead of indomethacin, was infused into the dog. Beta receptor blockade was considered effective if, at the end of the experiment, injection of 2 μg/kg of isoproterenol intravenously did not produce an increase in heart rate.

Major Findings and Significance:

Renal arterial infusion of BK (3 μg/min) in control dogs produced a sustained increase in urine flow rate (V), sodium excretion ($U_{Na}V$), potassium excretion (U_KV), and renal plasma flow (RPF) without a consistent change in glomerular filtration rate (GFR) or renin secretion rate (RSR). This increase in salt and water excretion and in RPF could not be blocked with indomethacin $U_{Na}V$ was 20.8 ± 7.4 and 123.3 μEq/min (Mean $\pm$ SEM), ($p<0.005$) before and after 140 min of infusion of BK. Beta-receptor blockade with propranolol did not prevent the BK-induced rise in salt and water excretion, or in RPF. $U_{Na}V$ was 26.0 ± 9.7 and 96.4 ± 21.5 μEq/min before and after 140 min of infusion of BK ($p<0.005$), respectively. The data suggest that the effects of BK on renal handling of salt and water and on RPF are not mediated by either prostaglandins or beta receptors.

Publications:

1. Brouhard, B.H., Gill, J.R., Jr., Yun, J.C.H., Kelly, G.D. and Bartter, F.C.: Prostaglandin dependent and independent effects of bradykinin on renal function in the dog. Renal Physiology 2: 44-53, 1979/1980.

2. Yun, J.C.H., Kelly, G.D., Bartter, F.C. and Tate, J.: Effect of indomethacin on renal function and plasma renin activity in conscious dogs. Renal Physiology 2: 295-301, 1979/1980.

3. Yun, J.C.H., Gill, J.R., Jr., Bartter, F.C., Kelly, G.D. and Keiser, H.R.: Effect of bradykinin on renal function in dogs treated with indomethacin or propranolol. Renal Physiology, in press.

ANNUAL REPORT
SECTION ON BIOCHEMICAL PHARMACOLOGY
HYPERTENSION-ENDOCRINE BRANCH
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

The primary direction of our research during the past year has remained unchanged. We continue to explore fundamental mechanisms of neuronal systems. The role of cyclic AMP and Ca^{++}-calmodulin dependent protein phosphorylations in these systems becomes increasingly a focal point of our research. One of the major long-term objectives of this work is to understand the participation of these neuronal systems in blood pressure regulation and certain neurological diseases.

I. Protein Phosphorylation in the Central Nervous System.

Neuronal cells in the central nervous system form complex networks of circuits to integrate and relay information. The fundamental chemistry of how these cells communicate has been the subject of intense investigation for many years. One putative mechanism for this information transfer is the rapid phosphorylation-dephosphorylation of membrane protein following interaction of a neurotransmitter from one cell with a receptor from an adjacent cell. One of the objectives of our work has been to characterize the protein kinases from brain and the endogenous substrates which accept the phosphate groups. One such kinase originally described by another research group appeared to exist in brain primarily as a proenzyme that could be activated by a proteolytic cleavage. A year ago we reported that this proenzyme could be activated by Ca^{++} under the appropriate assay conditions. It had also been reported in other laboratories that this enzyme could be activated by phospholipids in the presence of Ca^{++}. Although there are still many confusing issues about this protein our recent research has served to clarify our thinking about this enzyme.

(1) The proenzyme appears to belong to a growing list of enzymes that can be activated by limited proteolysis. However, the enzyme thus activated loses its normal regulatory mechanism, and this mode of activation may not be an endogenous mechanism.

(2) If the Mg^{++} concentration of the assay mixture is reduced to 4 mM the activity is stimulated by Ca^{++}. This Ca^{++} dependency can be demonstrated in the absence of phospholipids when a reducing agent (DTT) is present at a concentration of 30 mM, but is dependent upon phospholipids in the absence of DTT.

(3) The activation by Ca^{++} is inhibited by fluphenazine and other antipsychotics known to bind to calmodulin because of their hydrophobicity. The basal activity of this kinase is not inhibited by these agents.

(4) A major question is whether calmodulin is a subunit of the enzyme or whether the enzyme itself has a region which binds Ca^{++} in a manner anologous to calmodulin. Here our results are equivocal because of limited amounts of pure enzyme, however, a protein band has been observed on slab gel electrophoresis which would be consistent with a calmodulin subunit. A new HPLC analytical system currently being established in our laboratory will be used to resolve this problem.

A second type of calcium dependent protein kinase has also been under investigation in our laboratory. This protein kinase is present in the cytosol of synaptosomes isolated from rat brain. This enzyme(s) is remarkable in that it is responsible for the majority of endogenous phosphorylations that occur within synaptosomes and in this case it is totally dependent upon Ca^{++} and the Ca^{++} binding protein calmodulin. A cyclic AMP dependent kinase is also present and catalyzes the phosphorylation of several specific proteins. During the past year the development of a system to isolate large quantities of calmodulin and prepare large calmodulin-sepharose columns have aided us in several projects on calmodulin-dependent enzymes. With such a calmodulin sepharose column we found that a highly purified protein kinase from synaptosomes could be prepared quickly. This enzyme preparation, which initially had a complete dependency upon calmodulin and Ca^{++}, became only partially dependent upon Ca^{++}-calmodulin and no longer adsorbed to calmodulin-sepharose after overnight storage at 4°. This puzzling change in properties could be at least partially prevented by the addition of protease inhibitors. Clearly this enzyme, too, was very sensitive to endogenous proteases and its properties were being rapidly altered by very limited proteolysis. This observation probably explains why differing properties of this enzyme have been reported by various laboratories. Whether such proteolysis is a normal or abnormal physiologic reaction is not known.

In addition to the cytosol protein kinases described above there are membrane kinases. In the previous report we described a system of self phosphorylation for synaptic plasma membranes. This system appears to contain both cyclic nucleotide and calmodulin dependent protein kinases in addition to kinase(s) that are not regulated by either of the above regulatory systems. We reported a factor that stimulates protein phosphorylation of membrane protein by a mechanism that is not dependent upon either Ca^{++} or cAMP. We have attempted to characterize this factor with limited success. There are relatively few substrate proteins whose phosphorylation is enhanced by this factor suggesting that it operates in conjunction with a specific kinase. It can be distinguished from calmodulin since it is not adsorbed to fluphenazine-sepharose. Attempts to purify that factor have been unsuccessful. While it is possible that this factor is a phospholipid the lack of Ca^{++} dependency argues against this possibility. Work is continuing on this factor and the nature of the major substrate that is stimulated.

Part of our interest in the Ca^{++} dependent protein kinase stems from the possibility that it may be involved in the adaptive reactions that take place during the process of addiction to opiates. We have found that three major synaptic plasma membrane substrates for the membrane bound Ca^{++}-calmodulin kinase show enhanced phosphorylation _in vitro_ following _in vivo_ treatment with morphine. This effect is blocked by naloxone. These results can be interpreted two different ways.

a) Morphine causes a dephosphorylation of these protein _in vivo_ allowing for greater incorporation of $^{32}PO_4$ _in vitro_.

b) Morphine enhances the accumulation of calmodulin in the plasma membranes and in this manner allows for a more rapid phosphorylation of these substrates.

Current experiments are designed to directly evaluate the calmodulin content of synaptic plasma membranes after various periods of morphine administration.

II. Aromatic Amino Acid Hydroxylases:

The synthesis of serotonin and several catecholamines which serve as neurotransmitters is controlled in the brain by tryptophan and tyrosine hydroxylase respectively. A major impetus for the research described above on protein kinases of brain arose from our earlier discoveries that the activity of each of these hydroxylases is regulated by direct phosphorylation of the enzyme proteins. Although the mechanisms of activation for these two hydroxylases appear to be similar (i.e. an increase in affinity for the reduced cofactors) the activations are accomplished by two distinct protein kinase. The activation of tryptophan hydroxylase requires a Ca^{++}-calmodulin dependent protein kinase. The lability of tryptophan hydroxylase has precluded the complete isolation of this enzyme. However, calmodulin can be totally separated from the enzyme and its specific protein kinase by chromatography on a fluphenazine-sepharose column. This enzyme mixture is now totally dependent upon the readdition of Ca^{++} and calmodulin for activation, which in turn is powerfully inhibited by fluphenazine and other antipsychotics that bind to calmodulin. It is possible that some of these drugs exert their pharmacological effect by inhibiting the regulatory system for serotonin synthesis. Current studies are aimed at isolating the specific protein kinase that activates this enzyme. It is hoped that it is identical or very similar to the calmodulin-Ca^{++} dependent kinase described in the preceding section. The isolation of tryptophan hydroxylase and the direct demonstration of phosphate incorporation remains a challenging task. Of interest are our recent studies showing that this enzyme, while not being a calmodulin dependent enzyme, binds weakly to calmodulin-sepharose. This observation should prove extremely useful in the isolation of this labile enzyme. In regard to this lability, our studies showing the remarkable stability of this enzyme in oxygen-free solution suggest that we may have to work in anaerobic media for the isolation. Also of interest is the fact that the reduced cofactor stabilizes the enzyme possibly providing a further tool for an approach to isolation.

In contrast tyrosine hydroxylase is regulated by a cAMP-dependent protein kinase. Many of our contributions to the understanding of the important regulatory mechanism have been reported in prior years and will be covered in the next section of this report. Work during the past year has led to a new concept of how dopamine synthesis is controlled. It is clear that at physiologic pH and hydroxylase cofactor concentration the non-phosphorylated form of tyrosine hydroxylase is essentially inactive whereas the phosphorylated form is fully active. By examining the effect of cofactor concentration with and without phosphorylation, it is possible to kinetically describe these two forms of tyrosine hydroxylase. A similar examination of the enzyme in tissue extracts indicates that in both the adrenal and corpus striatum only about 20% of the enzyme is in the activated state. Therefore the majority of enzyme has very little activity and is far below saturation with regard to the hydroxylase cofactor. We have further demonstrated that tyrosine hydroxylase can be activated *in vivo* by pharmacological or physiological manipulation.

III. Studies on the Hydroxylase Cofactor

Both tryptophan and tyrosine hydroxylase require a reduced pterin as an electron donor for the hydroxylation reaction. The pterin that serves this role in mammals is thought to be tetrahydrobiopterin (THB), although synthetic analogues can participate in the enzymically catalyzed reaction. It is clear from our

above discussion that THB is an extremely important regulatory factor in catecholamine and serotonin biosynthesis. Research in our group has increasingly focused on the regulation of cofactor content of tissues, its synthetic pathway, and attempts to pharmacologically manipulate levels of cofactor activity in tissues.

In the previous year we reported that the hydroxylase cofactor was highly localized in brain regions having significant amounts of tyrosine and tryptophan hydroxylase, and that the cofactor content of CSF from patients with Parkinson's disease was reduced. Our objectives for the past year have been to set up systems for studying the biosynthesis of THB, the regulation of cellular content, and the effect of increasing intracellular concentrations on the hydroxylase reactions. The ultimate objective of this work is to determine the clinical effect of modifying tissue levels of hydroxylase cofactor in parkinsonian patients. In order to more accurately assess the role of THB in striatal dopamine synthesis it is necessary to know the concentration within dopamine neurons, and its relationship to the concentration of the cosubstrates and tyrosine hydroxylase. These studies employed rats with neurotoxic, unilateral lesions (6-hydroxydopamine) of the substantia nigra. This procedure should selectively destroy dopamine nerve terminals in the unilateral striatum without significantly affecting other neuronal or glial systems. This procedure resulted in 90% loss of tyrosine hydroxylase and about 70% loss of THB and GTP cyclohydrolase, the key enzyme in the THB biosynthetic pathway. With this data we were able to calculate that the cofactor and its biosynthetic machinery were located specifically within aminergic cells in the brain. Furthermore, based on some reasonable assumptions, we could calculate that the concentration of THB within terminals is about 100 μM. This calculation is of particular interest since one could also calculate that the concentration of tyrosine hydroxylase subunits in dopamine terminals is also 100 μM whereas that of tyrosine is only about 70 μM. Based on these findings and the result reported in the previous section it is evident that only 20% of the enzyme that is phosphorylated is catalytically active and that additional enzyme activity could be recruited if one could significantly increase THB levels in tissue. While current dogma indicates that tyrosine is not a limiting factor in catecholamine synthesis, it is apparent that if the enzyme is significantly activated the rate of transport of tyrosine into the terminal could become rate limiting. It should be noted that work recently reported in the literature supports this concept. Under control conditions the rate of dopamine synthesis is independent of plasma tyrosine content; whereas in animals in which tyrosine hydroxylase has been activated pharmacologically the rate of striatal dopamine synthesis was dependent upon plasma tyrosine. These studies have given us a new understanding of the molecular details of the synthesis of a specific neurotransmitter and also encourages us to pursue our research on regulation of THB levels.

As a model system for studying cellular regulation of THB we have taken two approaches (pineal gland in organ culture and cultured mast cells). These systems were selected because each tissue had an unusually high content of THB. The mast cell system could demonstrate increases in THB amounts although the time frame was such that cells were undergoing division and the regulation of individual cellular content was difficult to evaluate. The pineal system, however, has considerably more promise. In this case glands can be cultured for two to three days and the levels of reduced pterin in the cell and total pterin in the media monitored. Early experiments indicated that supplying the gland with GTP precursors, the ultimate precursor of THB, could not influence the rate of THB synthesis.

In another series of experiments designed to directly explore the effects of administering THB and synthetic analogues, we have measured the rate of penetration of these compounds into the central nervous system of rats. We find that THB is taken up poorly, but that the more hydrophilic molecule 6-methyltetrahydropterin rapidly enters the brain. However its effect on dopamine synthesis remains to be determined. It is apparent that the ultimate treatment of neurological diseases related to a possible loss of cofactor activity will require a more lipophilic molecule that is a good cosubstrate for the hydroxylases. In this light we have tested 6-phenyltetrahydroptein with each of the hydroxylases and found that it is kinetically similar to the natural cofactor. This compound should exhibit lipophilic behavior and should readily penetrate the blood-brain barrier. With this type of approach we hope to be able to stimulate neurotransmitter synthesis specifically within the appropriate neurons.

IV. Molecular Events Associated with Neurotransmitter Release and Receptor Activation.

The mechanism of information transmission between neurons and their target cells is via release of a transmitter substance that results with appropriate receptors. Several individual projects within our group address various aspects of this question.

Interaction of dopamine with its receptor in the central nervous system has long been known to activate an adenylate cyclase in the membrane. The molecular details of the reaction have been under intense investigation in many laboratories. The role of calcium in the formation and metabolism of cyclic nucleotides suggested a possible involvement of calmodulin in this coupling process. In last years report we described the development of a sensitive immunoassay for calmodulin. With this assay we have now been able to follow translocation of calmodulin between membranes and cytosol both _in vivo_ and _in vitro_. Long term treatment of rats with dopamine receptor blocking agents results in a significant accumulation of calmodulin in the membrane fraction of the striatum. Using standard procedures to measure the turnover rate of a protein it could be demonstrated that the accumulation was not due to an increased synthesis. Since there appeared to be little change in the cytostolic fraction, it appeared that receptor blockade altered the membrane binding mechanism making more protein available for extraction. Conversely acute receptor activation appears to result in a translocation of calmodulin from the membrane to the cytosolic fraction. Analysis of receptor characteristics in striatal membranes from rats subjected to long-term receptor blockade indicated an increase in number (B_{MAX}) and an increase in responsiveness of adenylate cyclase to dopamine agonist. The development of this supersensitivity appears to be associated with the observed increase in membrane bound calmodulin. In a further attempt to characterize the role of calmodulin in dopamine receptor function striatal slices were incubated for various times with dopamine receptor agonists. In these experiments there appeared to be a translocation of calmodulin from the membranes to the cytosolic fraction. Receptor binding studies indicated a reduction in B_{MAX} and a decrease in the sensitivity of the adenylate cyclase system to dopamine agonist. Analysis of the cytosolic fraction by molecular sieve chromatography showed that the released calmodulin was in part free and in part associated with a high molecular weight fraction presumably phosphodiesterase. These studies lead to two important conclusions:

1) Calmodulin appears to be intimately involved in dopamine receptor function and the development of super- and subsensitive states of the

receptor complex and

2) it is possible to use an _in vitro_ brain slice to demonstrate and study the phenomenon of subsensitivity.

Another approach to understanding intercellular communication has been to investigate interaction of neuronal systems. An example of this are our findings on the effect of cocaine on serotonin and Substance P neurons in the CNS. It has been suggested, but not proven, that Substance P and serotonin coinhibit certain neurons in the brain. Furthermore, it is known that cocaine can affect serotonin turnover, perhaps indirectly by inhibiting norepinephrine uptake. We find that treatment of rats with cocaine results in reciprocal changes in serotonin and Substance P concentration in certain Raphe nuclei, i.e., Substance P levels increased whereas serotonin levels decreased. The reverse was true following administration of 5-hydroxytryptophan. These findings provide yet another example of receptor mediated responses in multiple neurotransmitter systems.

The continuing studies on the mechanisms of the carotid body response to hypoxia added further light on the interaction of receptor systems. We previously reported that exposure of rats to hypoxic conditions results in a short-term release of dopamine, the major transmitter in the glomus cells, and a long-term induction of tyrosine hydroxylase. More recently we have found that the reaction is dependent upon activation of a muscarinic receptor. The carotid body thus senses changes in oxygen content of blood by a complex interaction of catecholaminergic and cholinergic systems. Of significant interest is the fact that long term exposure of the carotid body to hypoxic conditions results in dramatic increases in tyrosine hydroxylase, and dopamine resulting from hypertrophy and hyperplasia of the glomus cells.

V. Neuropeptides.

In the past two years increasing work in our laboratory has been directed toward understanding the role and interactions of Substance P and enkephalin with other neurotransmitter systems.

One of the major problems for any researcher in the neuropeptide field is having a sensitive, specific and rapid means to measure the material. Radioimmunoassays provide the basis for most analytical systems with specificity being determined by doing RIA following separation on HPLC. With Substance P as a model we have attempted to devise a new approach. This procedure involved the use of small columns containing monoclonal antibodies to Substance P coupled to sepharose for the isolation of the specific Substance P-like material. This Substance P could be eluted from these columns and quantified by a HPLC system. Theoretically this type could be developed for any neuropeptide and would permit both quantitation and identification of multiple samples. Work is still proceeding on this approach, but it has been possible to demonstrate its feasibility with Substance P determined in certain brain regions.

During the development of this new method we have continued to use the standard RIA for Substance P. We have recently completed a detailed study of the interaction of the striatal-nigral-substance P with the nigral-striatal dopamine system. In the corpus striatum it appears that dopamine terminals communicate with Substance P neurons whose terminals end in the substantia nigra on dopamine cell bodies. Release of Substance P serves as an exciting transmitter for the dopamine system. Our studies have added support to the above concept in a series

of experiments involving neurotoxin and mechanical lesions as well as receptor manipulation. It now appears likely that the Substance P system performs a major regulatory role for the nigral-striatal dopamine system. In a developmental study in rats we find that the Substance P neuronal system is already well developed in 15 day old embryos. We are also continuing our work on the potential differences in the Substance P system normotensive and hypertensive rats.

Studies in several laboratories have identified enkephalin-like peptides and protein precursors of these peptides in the adrenal medulla. Little is known of the physiologic role of these peptides and their mechanism of secretion. We have studied the content of met enkephalin-like peptides in adrenal gland and in the adrenal venous outflow in dogs after electrical stimulation of splanchnic nerve or direct stimulation of nicotinic or muscarinic receptors of the adrenal medulla. Electrical stimulation resulted in a voltage dependent rise in plasma enkephalin-like peptide content. This secretion was blocked by hexamethoninium and mimicked by administration of nicotinic agonists. Analysis of the plasma shows that the stimulated secretion caused a rise primarily in the low molecular weight fraction of enkephalin-like material. The physiologic role of these adrenal peptides is not known, however in dogs that were pretreated with reserpine to deplete adrenal catecholamine stores splanchnic stimulation resulted in a hypotensive response. Studies are continuing to understand the role of these neuropeptides in blood pressure regulation.

VI. Neuronal System and Blood Pressure Regulation.

We have a standing interest in central neuronal systems and blood pressure regulation. Our interest has focused primarily on the catecholamine and serotonin neuronal systems. In our prior report we demonstrated that electrical stimulation of serotonin cell bodies in the dorsal and medial Raphe nuclei results in a marked pressor response. This study identified the effective neurons as serotonergic and suggested that the information was directed in an anterior direction perhaps through the anterior hypothalamus before being directed to the periphery. In the current work it is of interest to note that this serotonergic pressor system is also functional in the spontaneously hypertensive rat (SHR) and the control Wistar-Kyoto (SKY). In an effort to further trace potential serotonergic pathways, direct micro-injection of serotonin was applied to discreet brain regions. The nucleus tractus solitarius receives rich serotonergic innervation and is known to play a central role in blood pressure regulation. Injection of 2 to 30 nmol of serotonin resulted in an increase in blood pressure. This effect could be blocked by serotonin antagonists. Similar injection into the preoptic area and anterior hypothalamus results in similar blood pressure responses. This suggests that neurons mediating responses are directed to both the anterior hypothalamus and the NTS from the Raphe nuclei. The significance of these findings is that central serotonin neurons play an important role in cardiovascular responses.

For a number of years we have been investigating depressor responses that appear to mediate noradrenergic systems in the brain. Considerable evidence for such systems now exists and some of the major antihypertensive agents appear to act by the stimulation of central α receptors. Our studies on the regulation of tyrosine hydroxylase <u>vide supra</u>, and a report in the literature suggested that under certain conditions it might be possible to enhance catecholamine synthesis by increasing the availability of tyrosine. In some relatively simple experiments we found that the intraventricular administration of 15 μg of tyrosine caused a significant reduction in blood pressure of hypertensive rats and simultaneously

increased the turnover of norepinephrine in the brain of these rats. It appears likely that increased stimulation of α receptors in the CNS is responsible for the reduction in blood pressure. It should be noted in our previous report that the hypotensive agent Prazosin also stimulated norepinephrine turnover in a similar manner. Clearly, at least certain noradrenergic systems in the brain play a role in reducing blood pressure.

Epinephrine-containing neurons in the brainstem may also have a role in modulating blood pressure possibly through the "baroreflex". Last year we reported that the genetically hypertensive rats have a greater number of epinephrine containing cells in the C_1 and C_2 region of the brainstem. No experiments have yet proven that these neurons play a role in blood pressure regulation. The following line of experimentation moves us a step nearer to that determination. Analysis of PNMT, a marker enzyme for epinephrine neurons, shows about 25-30 percent more activity in SHR over WKY. If animals from either strain are subjected to unilateral carotid ligation below the bifurcation and PNMT activity measured 6 hours later on the right and left side of the brainstem, the PNMT activity is significantly increased on the contralateral side. Since most fibers involved in the baroreflex are thought to cross the midline, it would appear that loss of pressure and flow above the ligation cause epinephrine cells to become activated possibly as part of the baroflex attempt to overcome the loss of pressure. Considerably more experiments must be done although this is the first demonstration that epinephrine neurons may be involved in blood pressure regulation.

VII. Genetic Models of Hypertensive Diseases.

For a number of years we have studied the animal models for hypertension (SHR) and stroke (stroke-prone SHR). In last years report we demonstrated that for stroke-prone SHR in addition to hypertension, a permissive diet (low-protein) was necessary for the pathological event to occur. This important interaction between genetic and environmental (diet) factors is still under collaborative study, but there are no new observations to report this year.

We have, however, completed a detailed genetic and physiological study on the cause of cardiac hypertrophy in hypertensive rats. Twenty inbred strains of rats were studied at various ages from 4 to 16 weeks for the relationship between blood pressure and heart size. These strains had a wide range of "normal" blood pressure and heart sizes. Statistical analysis of that data indicated that genetic factors not related to blood pressure accounted for about 60% of the enlargement. This is true for both the left and right ventricle as well as the aorta. To determine whether heart size was due to hypertrophy of muscle cell or hyperplasia of non-muscle cells. DNA and RNA analysis were done on all the strains. As might be expected, a large proportion of the increase in size in hypertensive animals is due to a hypertrophy of the myocytes whereas the genetic heart size determinants are expressed as a hyperplastic response.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01843-08 HE

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Mechanisms of Storage, Uptake and Release of Norepinephrine in Adrenergic Nerve Endings (RT)

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

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Hypertension-Endocrine Branch

SECTION
Biochemical Pharmacology

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Additional details of the processes involved in the Choline (Ch^+) Ca^{++} stimulated, model amine secreting system in adrenergic nerve endings were discovered. The establishment of desipramine or cocaine evoked inhibition of Ch^+-Ca^{++} stimulated secretion required Na^+ in the external medium, but did not require Na^+ to remain in effect. The inhibitors prevented release and stopped on-going release, provided that Na^+ was supplied. Potassium prevented the development of inhibition, but did not relieve an established block of Ch^+-Ca^{++} stimulated release. The inhibitors did not block the secretion induced by K^+ added to Krebs (KRB) or to the Ch^+-Ca^{++} medium. Thus, the secretions evoked by each of the two stimuli were independent of the other, but involved the same synaptic vesicles. In vivo depolarization would inhibit the Na^+ dependent attachment of desipramine to its receptor. Additionally, more recent results on the inhibition of Ch^+-Ca^{++} stimulated secretion by energy conserving nucleotides paralleled the results of published experiment with isolated synaptic vesicles (Euler) more closely than previous experiments. The interaction between ATP and Ca^{++} was eliminated as a possible mechanism of ATP induced inhibition.

PHS-6040
(Rev. 2-81)

Objectives: To investigate the possibility that transmitter amines in adrenergic nerve endings could be released by a process other than exocytosis or diffusion. Specifically, the following questions where addressed: Are the amines secreted in response to Ch^+-Ca^{++} stimulation released from synaptic vesicles attached to the plasma membrane in proximity to the transport mechanism? What are the ionic conditions required for the inhibition of release by inhibitors of transport? Are Ch^+-Ca^{++} induced release and depolarization induced release mediated by different channels? What might be the possible physiological significance of these experimentally induced secretions?

Methods: The objectives of this study were pursued by methods previously reported in detail.

Major Findings: Previous reports have shown that Ch^+-Ca^{++} stimulated release is mediated by outward transport of amines. The reports suggested that the transported amine was located in synaptic vesicles attached to the plasma membrane at a site close to the transport mechanism. In previous experiments to show that transport inhibitors block Ch^+-Ca^{++} stimulated release, the tissues have routinely been exposed to inhibitors during the final 15 minutes of preincubation of the freshly prepared slices in a Krebs-bicarbonate medium (KRB). The slices were then transferred to the Ch^+-Ca^{++} stimulating medium containing the inhibitor. A question that has arisen as a result of these studies is whether the Ch^+-Ca^{++} stimulated release would be blocked when first challenged during the phase of rapid release. A series of experiments studied this question. The basic experiments were varied by using 5 ml of incubation medium and changing this medium every 20 min. In this way, the tissues were exposed to the inhibitor, as well as various electrolytes, during the 20 min interval between the 80th and 100th min of incubation, at which time the rate of release had become rapid. The inclusion of desipramine in the medium at this time had little release blocking effect. The possibility of a Na^+-dependency for release blockade, by analogy with Na^+-dependent transport of amines, was then tested by a variety of experiments in which the Na^+ concentration was raised above the 18 mM concentration in the Ch^+-Ca^{++} release stimulating medium. These higher concentrations of Na^+, in themselves inhibited Ch^+-Ca^{++} stimulation. Desipramine induced a slightly greater inhibition of Ch^+-Ca^{++} stimulated release of amines. Whereas the Na^+-induced inhibition of the release of amines did not outlast the presence of elevated concentrations of Na^+, the presence of desipramine powerfully inhibited release even after the Na^+ was eliminated from the media. Thus, the release-inhibiting effect of desipramine was Na^+-dependent to establish inhibition, but was independent of Na^+ once the inhibition was established.

The Na^+-dependent, desipramine induced inhibition of Ch^+-Ca^{++} stimulated release was induced whether the Ch^+-Ca^{++} medium was replaced with KRB containing desipramine, or whether the desipramine, together with Na^+, was added to the Ch^+-Ca^{++} stimulating medium.

It was tentatively concluded that Ch^+-Ca^{++} stimulated release depends upon the loss of intracellular Na^+. However, choline itself prevented the release induced by the Na^+ deprivation. Thus, the release of amines induced by media in which the Na^+ was replaced by sucrose or Li^+ began immediately upon immersion of the tissues in these media, and did not require Ca^{++}. Moreover, the addition of a low concentration of choline (55 mM) to a sucrose medium inhibited release whether or not Ca^{++} was present in the medium. Cocaine did not block this action of choline.

It is therefore concluded that the delayed $Ch^{+}-Ca^{++}$ stimulated release also depends upon the entrance of Ca^{++} into the Na^{+}-depleted nerve ending. Thus, the release of amines can be reversed by the addition of Na^{+} to the $Ch^{+}-Ca^{++}$ stimulating medium. The Na^{+} apparently antagonizes the amine-releasing effect of Ca^{++} that has entered the nerve ending during $Ch^{+}-Ca^{++}$ stimulation. This physiological relationship between Na^{+} and Ca^{++} was postulated by Bogdanski in 1973.

The fact that the Na^{+}-dependent effect of desipramine outlasted the presence of Na^{+} suggested that the inhibition of release was the result of a Na^{+} dependent attachment of desipramine to a receptor, or carrier, or that desipramine inhibited release after its transport to the interior of the nerve ending.

The possibility that Na^{+} by itself slowed $Ch^{+}-Ca^{++}$ stimulated release by stimulating the uptake of released amine was eliminated by the experiments with desipramine. Desipramine would have increased release by blocking the Na^{+}-dependent uptake while both were present.

Previous reports have postulated that outward transport of amines may be involved with synaptic transmission. The possible interaction between K^{+} and the Na^{+}-dependent, desipramine mediated inhibition of $Ch^{+}-Ca^{++}$ stimulated release was, therefore, studied. Besides providing basic information, the studies could provide an explanation for the weak or negative response of synaptic transmission to the presence of transport inhibitors. In a set of experiments devised to study this problem, the $Ch^{+}-Ca^{++}$ stimulated secretory response was allowed to develop up to the 80 min time point, and then the medium was replaced with a medium containing approximately equimolar concentrations of Na^{+} and K^{+}, with and without desipramine. Potassium stimulated amine secretion but the effect of K^{+} was not inhibited by desipramine. Upon the return of the slices to the $Ch^{+}-Ca^{++}$ stimulating medium with and without desipramine, the K^{+} stimulated amine release was eliminated leaving the $Ch^{+}-Ca^{++}$ stimulated release of amines. This release was not inhibited by the desipramine. Thus, K^{+} inhibited the Na^{+}-dependent release blocking effect of desipramine.

In a second set of related experiments slices were preincubated in KRB with desipramine and then transferred to the $Ch-Ca^{++}$ medium. Release was inhibited, as usual. When the slices were transferred to a $Ch^{+}-Ca^{++}$ medium containing, in addition, K^{+} at a concentration of 66 mM, release was stimulated. Upon the return of the slices to the $Ch^{+}-Ca^{++}$ medium containing control concentrations of K^{+}, the K^{+} stimulated release was eliminated and the release rate was characteristic of desipramine-inhibited, $Ch^{+}-Ca^{++}$ stimulated release. Thus, desipramine did not inhibit K^{+}-stimulated release, but K^{+} did not eliminate an established inhibition of $Ch^{+}-Ca^{++}$ stimulated release. However, K^{+} prevented the establishment of Na^{+}-dependent, desipramine induced inhibition of $Ch^{+}-Ca^{++}$ stimulated release. It was tentatively concluded that K^{+} prevented the Na^{+} dependent attachment of desipramine to its receptor or prevented the transport of desipramine. It was assumed that this effect of K^{+} was analogous to the K^{+} induced inhibition of Na^{+}-dependent transport of amines.

These results of experiments and conclusions are in accord with the postulates of Bogdanski, 1975, which were proposed to explain the failure of transport inhibitors to block synaptic transmission if that process were mediated by the outward transport of amines. Thus, K^{+} induced depolarization at least partly mimics the

physiological depolarization of nerve endings. Moreover, nerve activity increases the local concentration of K^+ at the external side of the plasma membrane, and, the resting potential itself is mainly a diffusion potential developed by intracellular K^+. All these factors may combine to inhibit the effect of desipramine on the outward transport of amines.

The lack of effectiveness of desipramine against K^+ at a time when Ch^+-Ca^{++} stimulated release was already established, and the resumption of inhibition of Ch^+-Ca^{++} release after the K^+ was removed, suggests that desipramine would not strongly inhibit release initiated electrically, or by other rapidly induced depolarization (as opposed to the slow depolarization induced by Na^+ deprived media). As we have postulated, however, K^+ induced release can occur in nerves whose vesicles have been oriented to a membrane transport carrier by Ch^+-Ca^{++} stimulation. In the converse situation, the vesicle oriented to the membrane by depolarization may also release amines through the transport channel. These conclusions are in accord with the hypothesis proposed by Blaszkowski and Bogdanski, 1972, and Bogdanski, 1975. In any event, the Ca^{++} dependent orientation of vesicles to the plasma membrane may be the same for both cases.

The problem of whether or not amines were released from vesicles attached to the plasma membrane was addressed by using the same approach as described in a previous report. This approach involved the ability of exogenous, extracellular, energy-conserving nucleotides to inhibit fully-established, Ch^+-Ca^{++} stimulated release. Previous experiments had suggested a monovalent electrolyte dependency for the release inhibiting effects of ATP. In the present experiments, care was taken to compare the effects of nucleotides when the low concentrations of Na^+ and K were held constant. The acidity of nucleotide solutions was neutrallized with $NaHCO_3$, $KHCO_3$ in place of KCl and with choline bicarbonate in place of KOH. The pH was adjusted to a slightly lower range of 7.2 ± 0.05. The results of these experiments indicated that ATP was more potent than UTP as an inhibitor of Ch^+-Ca^{++} stimulated release. Previous experiments had shown the opposite. Moreover, ADP was a weaker inhibitor of release than suggested by previous experiments. These findings correspond more closely to the results reported by Eiler's laboratory for their studies on isolated nerve vesicles. Perhaps more significant, however, is the consistency of the finding that the nucleotides inhibit Ch^+-Ca^{++} stimulated release but have no effect on the uptake of ^{3}H-NE by slices incubated in KRB. In a previous report, the release inhibiting effect of ATP has been interpreted as being the result of stimulated uptake of NE by vesicles attached to the plasma membrane during Ch^+-Ca^{++} stimulation.

The possibility remained, however, that ATP inhibited Ch^+-Ca^{++} stimulated release by eliminating Ca^{++} from the reaction. This problem was studied by pre-mixing ATP with Mg^{++} before introducing the ATP-Mg^{++} to the Ca^{++} containing medium. The concentration of Mg^{++} used was 2.36 mM (2 x control) whereas the concentration of Ca^{++} used was 1.27 mM (0.5 x control). The concentration of ATP was 3 mM. In some biochemical reactions ATP is known to act through the Mg^{++} complexed form. In the experiments for this report, the ATP-Mg^{++} complex should decrease the ATP induced inhibition of Ch^+-Ca^{++} release by interfering with the ATP complexing with Ca^{++}. As previously found, however, Mg^{++} actually increased the release inhibiting effect of ATP. This effect was in accord with actions reported by Euler for his studies on isolated splenic nerve vesicles. For the vesicle membrane to be accessible to extracellular ATP-Mg^{++} in Ch^+-Ca^{++} stimulated tissues, the vesicle would most likely be attached to the plasma membrane. The membranes at this site

remain closed, not open as in exocytosis, as shown by the Na^+-dependent, desipramine induced inhibition of ongoing Ch^+-Ca^{++} stimulated release. It is considered more likely that desipramine attached to a receptor mediating release than that desipramine closed off a site of exocytosis.

Preliminary experiments addressed the question of whether a calmodulin dependent system was involved in the Ch^+-Ca^{++} stimulated release. The essential findings were these: Fluphenazine, in concentrations ranging from 1 to 60 μg/ml evoked a concentration dependent release of ^3H compounds in slices incubated in the KRB and increased the release of amines in Ch^+-Ca^{++} stimulated tissues including those given ATP.

As stated above, the effect of ATP was sensitive to the presence of Na^+ and K^+ in concentrations that significantly differed from the control concentrations. This monovalent cation dependency of ATP induced inhibition of Ch^+-Ca^{++} stimulated release of amines suggested that phosphorylation reactions were involved in the release. Such reactions, if they occurred, did not appear to be initiated by calmodulin dependent reactions, nor inhibited by ouabain. The release was not inhibited by probenecid, an inhibitor of anion transport.

The possible relationship between Ch^+-Ca^{++} stimulation and nicotinic stimulation was studied briefly. Nicotinic stimulation by acetylcholine-atropine did not evoke a detectable release by our methods. In any event, the delayed, prolonged and massive release of amines induced by Ch^+-Ca^{++} stimulation differs from the immediate, transient and low-quantity release of amines induced by nicotinic stimulation . Atropine had no effect on Ch^+-Ca^{++} stimulated release.

Further studies on slices exposed to the autodialysis technique (reported previously) reaffirmed the viability of such tissues and presence of normal processes involved with the storage and release of biogenic amines. Thus, reserpine strongly depleted the ^3H-NE in dialyzed slices subsequently incubated in KRB. This finding indicated that the amines were stored in synaptic vesicles in these tissues.

Significance to Biomedical Research and Institute Programs: The possible significance of these studies to an understanding of basic physiological processes is the fact that there is increasing evidence that exocytosis is not the only mechanism of secretion. The release processes in slices incubated in Na^+-deficient media ($choline^+$) containing Ca^{++} show numerous parallels to neurotransmission as discussed in earlier reports. Hence, our findings are relevant to neurosecretion and synaptic transmission. Our conclusions outlined above indicate the existence of specific Ca^{++}-dependent processes for transporting binding sites to the plasma membrane and suggest that mobilization and transport of NE may be intimately associated and possible are mediated by the same mechanism. The evidence now suggests that the vesicle and plasma membranes coalesce to form a new structure which retains the NE transporting mechanisms of both membranes. During the process of coalescence, vesicle membrane DBH and ATP could be released to the extracellular medium, but not in quantities expected if release were mediated by exocytosis.

Proposed Course of Project: The basic structure of the model neurosecretory system has now been expressed, and parallels are being established between this model and known mechanisms. The establishment of a new membrane structure incorporating elements of the plasma membrane and synaptic vesicles would substantiate physiological significance.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01846-07 HE

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Molecular Biology to Chemoreceptor Regulation

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

PI:	Ingeborg Hanbauer	Pharmacologist	HE NHLBI
OTHER:	Farouk Karoum	Pharmacologist	LCP NIMH
	Sukhamay Lahiri	Professor	University of Pennsylvania

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Acute hypoxia causes a selective release of dopamine from glomus cells without altering the norepinephrine stores in carotid body. A similar pattern of changes in catecholamine content is elicited by the injection of muscarinic receptor agonists. Methylatropine prevents the release of dopamine elicited by methocholine and by acute hypoxia. These findings suggest that a cholinergic muscarinic receptor may mediate dopamine release. In contrast, long lasting hypoxia increased dopamine and norepinephrine content. This response appears to involve induction of tyrosine hydroxylase and hypertrophy and for hyperplasia of glomus cells.

PHS-6040
(Rev. 2-81)

Objectives: The carotid body is a chemoreceptor organ which senses changes in the partial pressure of arterial blood gases. In mammals the regulation of the acute response to hypoxia is triggered by stimulation of chemoreceptors located in the carotid body. During long-lasting hypoxia compensatory changes in circulation, rate of respiration and pulmonary gas exchange are greatly reduced in the absence of carotid body chemoreceptors. The glomus cell is the most prevalent cell type in the carotid body and may be involved in the control of chemoreceptor afferent discharge. Dopamine was shown to be the major neurotransmitter in these cells, whereas norepinephrine is primarily located in sympathetic nerve endings of the carotid body vasculature. It is of interest to determine whether in carotid body the catecholamine content of glomus cells or of the perivascular sympathetic nerve endings is affected by physiological or pharmacological stimuli.

Methods: Long-term exposure to hypoxia. Three rats per cage were placed in a chamber in which the gas composition was maintained at 10% O_2 + 90% N_2 for various time periods ranging from 2 days to 4 weeks.

In some studies transection of the carotid sinus serve, or superior cervical ganglionectomy was performed unilaterally 5 to 7 days before the experiment.

Assay for catecholamines and their metabolites. The concentrations of dopamine, norepinephrine, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were measured by gas chromatography-mass fragmentography. The carotid bodies were homogenized in 0.1 N formic acid and deuterated analogues of the catecholamines and metabolites were used as internal standards. The turnover-rate of dopamine was determined by measuring the initial decline of the DOPAC content after injection of pargyline.

Major Findings: Modification of norepinephrine content by carotid body denervation. Transection of the carotid sinus nerve 7 days prior to the experiment increases the norepinephrine content, whereas removal of the superior cervical ganglion decreases it. In contrast, neither of the two types of denervation changes the dopamine content. These results suggest that dopamine is solely contained in glomus cells and the maintenance of its storage is independent from the carotid body innervation. The rise of the norepinephrine compartment in carotid body appears to be regulated by sensory and sympathetic renervation.

Effect of methacholine on dopamine storage in carotid body. Injection of methacholine (12.5 μmol/kg) decreases the dopamine content of rat carotid body without changing the norepinephrine content. This decrease in dopamine content is due to an increased rate of release of this neurotransmitter and also occurs after transection of the carotid sinus nerve or ganglionectomy. Methylatropine (21 μmol/kg) prevents the decrease in dopamine content elicited by methacholine. Since methylatropine could block also the decrease of dopamine content elicited by short-term hypoxia it is inferred that a muscarinic receptor may play an important role in the mechanism, whereby the carotid body senses changes in arterial blood gases. In fact, the release of dopamine elicited by exposure to hypoxia can be curtailed by atropine.

Increase of dopamine and norepinephrine content by long-lasting exposure to hypoxia. Exposure to hypoxia lasting for 2 days increases the dopamine content in rat carotid body, but fails to change that of norepinephrine. Exposure to hypoxia lasting one week increases the content of both dopamine and norepinephrine. The levels of both amines increase even further, when the exposure time to hypoxia is extended to 2 and 4 weeks. Denervation of the carotid body fails to prevent this increase. The specificity of these changes in carotid body catecholamine content is upheld by the finding that exposure to hypoxia for 4 weeks fails to change the catecholamine content in caudate nucleus, heart, superior cervical ganglia and adrenal glands.

Significance to Biomedical Research and Institute Program: The arterial chemoreceptors play an important role in the respiratory and circulatory response to hypoxia, hypercapria or metabolic acidosis. One of the main problems in carotid body function is to understand the mechanism whereby chemosensory impulses are initiated. To analyze physiologically and biochemically the events occurring at the receptor level, it is necessary to know how the carotid body is structurally organized and whether there are specific neurotransmitters involved in the chemosensoty response. Our studies reveal that dopamine present in the glomus cell is involved in the chemoreceptor response during short- and long-lasting exposure to hypoxia.

Proposed Course of Project: The following experimental approach is planned to improve our understanding of the biochemical mechanisms involved in chemoreceptor function.

(1) Studies on the effect of hypercapnia and metabolic acidosis on the dopamine and norepinephrine content of rat carotid bodies.

(2) Measurement of the peptide hormones - metenkephaline and vasoactive intestinal peptide - in carotid bodies of dogs during exposure to hypoxia or hypercapnia.

Publications:

1. Hanbauer, I., Karoum, F., Hellstrom, S., and Lahori, S.: Effects of hypoxia lasting up to one month on the catecholamine content in rat carotid body. Neuroscience 6:81-86, 1981.

2. Hanbauer, I.: Catecholamines and rat carotid body function. Adv. Physiol. Sci., Vol. 10, Respiration (I. Hutas and L.A. Debreezeni eds.) pp. 425-434, Pergamon Press, Oxford, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01847-07 HE

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Functional Role of Calmodulin 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

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	Sikta Pradhan	Guest Worker	HE NHLBI

COOPERATING UNITS (if any)

None

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SECTION
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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)

Persistent stimulation of striatal dopamine receptors increases the calmodulin content in the supernatant fraction and decreases it in the membrane fraction. This translation of calmodulin appears to be rate-limiting in protein kinase. Therefore, this protein can be viewed as an important component involved in determining dopamine receptor sub- and supersensitivity.

PHS-6040
(Rev. 2-81)

Objectives: Since in the CNS a complex system of heterogenous inter-connected neurons and associated neuroglia is operative, the participation of cyclic-nucleotides in the synaptic transmission must be studied along tactical lines. The goal of this study was to obtain information in the molecular mechanisms underlying the stimulation of dopamine receptors. Dopamine receptors, like many other post-synaptic receptors function as supramolecular entities and receptor activity is generated by interaction of a number of membrane proteins. In striatum, the occupancy of dopamine recognition sites activates adenylate cyclase. A specific GTP-binding protein appears to be generative in the couplings of adenylate cyclase to the dopamine recognition site. Also, other proteins such as calmodulin have been implicated in dopamine receptor function.

The present experiments were carried out to evaluate the participation of calmodulin the function of striatal dopamine receptors. It was of interest to establish the changes in the various components of the dopamine receptor during sub- and supersensitivity of the receptor. Moreover, since it has been described that antipsychotics bind with high affinity to calmodulin which may be related to the development of dopamine receptor supersensitivity. We studied also the dopamine receptor in striate and nucleus from control and schizophrenic brains.

Methods: Rat caudate nuclei slices were incubated in Krebs-Ringer solution pH 7.4 supplemented with ascorbic acid and dextrose. After preincubation for 60 min the drug under study was added and the incubation was continued for 30 min. The slices were then drained and homogenized in 0.32 M sucrose. After centrifugation at 4000 rpm for 10 min the supernatant was removed and recentrifuged at 39.000 rmp for 30 min. The pellet-fraction was extracted with Tris-HCl buffer pH 7.4 containing 0.1% lubrol or n-octylglucosid.

The kinetic properties of cAMP-phosphodiesterase were studied in the supernatant fraction of striatal homogenates. The content of calmodulin was estimated by micro-ELISA in both supernatant fraction and pellet-extract prepared from striatal homogenates. Specific ligand binding to striatal dopamine receptors was determined in striatal membrane preparations as described by Creese et al. (Durop. J. Pharmacol. 56:411-412, 1979). Adenylate cyclase activity in striatal homogenates was measured as described by Clement-Courrier (Proc. Natl. Acad. Sci., USA, 71:1113-1117, 1974).

Major Findings: 1. Role of calmodulin during desensitization of striatal dopamine receptors. Incubation of striatal slices with apomorphine (10^{-7} M) or dopamine (2×10^{-7} M) increases the calmodulin content in the supernatant fraction prepared from striatal homogenates. The amount of calmodulin associated with cAMP-PDE is increased after persistent stimulation of dopamine receptors.

In contrast, the calmodulin content in striatal membranes is significantly decreased. Furthermore, prolonged occupancy of striatal dopamine receptors reduces the dopamine-induced activation of adenylate cyclase. This loss in enzyme responsiveness to dopamine stimulation is associated with a decrease in membrane-bound calmodulin content and a loss in efficiency in the coupling system between detector site and adenylate cyclase.

2. Role of calmodulin during striatal dopamine receptor supersensitivity. Long-term treatment with haloperidol (1 mg/kg, 21 days) or injection of cocaine

causes supersensitivity of striatal dopamine receptors. This change in receptor sensitivity is associated with an increase in Bmax for spiroperidol or N-propylnorapomorphine, an increase in membrane-bound calmodulin content and an enhanced responsiveness of adenylate cyclase to dopamine activation. The supersensitivity of adenylate cyclase may be due to the increased availability of calmodulin in postsynaptic membranes.

Significance to Biomedical Research and Institute Programs: Considerable attention has been paid to cyclic nucleotides and their regulation in various tissues and various pathological states. Since cAMP participates as a second messenger linking receptor activity to biochemical processes, our studies on the response to dopamine receptor stimulation are of interest for the clarification of the action mechanism of neuroleptic and narcotic drugs.

Proposed Course of Project The functional role of second messengers on the regulation of neurotransmitter responses will be extended to other neuronal systems, which form synaptic interconnections with the striatal dopaminergic neurons. The input on dopamine receptor regulation by neuropeptides, such as cholecystekinin, met-enkephalin and neurotensin will be studied using calmodulin, adenylate cyclase, GTP-binding protein and GTPese as biochemical indices.

Publications:

1. Hanbauer, I.: Calmodulin in dopamine receptor function. In: Psychopharmacology and Biochemistry of Neurotransmitter Receptors. Yamamura, H., Olson, R. and Usdin, E., eds.), pp 475-482, Elsevier/North Holland, New York (1980).

2. Hanbauer, I. and Costa, E.: Role of calmodulin in dopaminergic transmission. In: Calcium and Cell Function Calmodulin (Cheung, W.Y., ed.), Vol. 1, pp. 253-272, Academic Press, New York (1980).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01850-12 HE

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Biochemistry of the Spontaneously Hypertensive Rat

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

PI:	Dr. M. Fujiwara	Visiting Fellow	HE NHLBI
OTHER:	Dr. Walter Lovenberg	Chief, Sec. Biochem. Pharmacol.	HE NHLBI
	Dr. Yukio Yamori	Guest Worker	HE NHLBI
	Dr. Ryochi Horie	Guest Worker	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.8	0.2

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The role of protein metabolism in the vasculature has been investigated with regard to the pathogenesis of hypertension and stroke in genetic animal models. Our previous observation was that incorporation of amino acids into vascular proteins in young spontaneously hypertensive rats was significantly increased. In an attempt to work with biochemical preparations, we prepared brain microvessels from rats that had been injected with radioactive lysine and examined incorporation into collagen and non-collagen proteins. The incorporation into microvessels was greater on a weight basis than in other small vessels and the SHR exhibited a higher rate of incorporation into collagen than normotensive controls.

PHS-6040
(Rev. 2-81)

Objectives: Vascular protein synthesis and wall structure may be important parameters in the pathogenesis of stroke and hypertension. In prior years we had shown that the rate of amino acid incorporation into peripheral vascular protein of hypertensive animals is increased. The object of work in the current year is to determine whether cerebral vessels isolated by biochemical techniques demonstrate the same phenomenon and whether this vascular protein synthesis in any way relates to the incidence of stroke in the genetically stroke-prone rats. We also wished to determine the effect of dietary protein on the incidence of stroke in stroke-prone animals.

Methods: Cerebral microvessels were isolated by modifications of established techniques. Incorporation of radioactive lysine into the protein of these vessels was measured 2 hours after a pulse injection. Blood pressures were monitored by tact-cuff plethysmography. For nutritional experiments, animals were obtained either from NIH small animal section or the Stroke-Prevention Center in Izumo, Japan. Starting at 6 weeks of age, animals were maintained in randomly divided groups for up to 10 months of age. At this age, animals which had not succumbed to stroke were sacrificed and their brains examined histologically.

Major Findings: The incorporation of ^{3}H-Lysine into collagen protein is approximately 3 times higher in brain microvessels and piae arteries than in the mesenteric artery and aorta.

The rate of ^{3}H-Lysine incorporation in 10-week old normotensive Wistar-kyato rat (WKY:PB 122 mm Hg) was as follows: in brain microvessel, piae artery, mesenteric artery and aorta, 140.1, 1271.8, 467.3 and 280.6 CPM/mg protein, respectively.

Incorporation of lysine was significantly higher in spontaneously hypertensive rats (SHR:BP 172 mm Hg) and in stroke-prone spontaneously hypertensive rats (SHRSP:BP 185 mm Hg) compared to WKY. The lysine incorporation into non-collagenous protein and elastin was not significantly changed.

From these studies it appears that increase of protein synthesis is greater in smaller vessels and in development of hypertension with age.

Significance to Biomedical Research and Institute Programs: The identification of risk factors associated with cardiovascular disease is one of the prime objectives of the NHLBI. Based on our studies with the stroke-prone spontaneously hypertensive rat it would appear that while hypertension is one of the major risk factors for stroke, this factor appears to be modified by the dietary protein intake. If these findings can be extended to man, a preliminary epidemiologic study in Japan supports this effect, than our view on the prevention of stroke will be altered.

Proposed Course of Project: In the coming year, we plan to examine the effect of dietary protein on the rate of protein synthesis in cerebral microvessels.

Publications:

Yamori, Y., Nara, Y., Horie, R., Ooshima, A. and Lovenberg, W.: Pathophysiological role of taurine in blood pressure regulation in stroke-prone spontaneously hypertensive rats (SHR). In: The Action of Taurine on Excitable Tissue, (S. Schaffer and S. Baskin, eds.) Spectrum Press, N.Y., 1979

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01851-07 HE

PERIOD COVERED
October 1, 1980 through September 30, 1981

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:	Judith Juskevich	Staff Fellow	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. on Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The primary goal of these studies is an understanding of the regulation of tyrosine hydroxylase in the central nervous system. Previously we have shown that phosphorylation or catecholamine removal activates the soluble enzyme. However, other factors may regulate tyrosine hydroxylase through mechanisms requiring the in vivo environment. Therefore, we are studying the rate of tyrosine hydroxylation in a synaptosomal system. Synaptosomes prepared from mouse whole brain, rat striatum and rat cortex have been used to study the regulation of tyrosine hydroxylase. Addition of calcium to the media increases tyrosine hydroxylase activity in synaptosomes from mouse whole brain and rat cortex, but not rat striatum. In addition, we have found that addition of calcium changes the kinetics of tyrosine hydroxylase for tyrosine in whole brain and cortical synaptosomes. Tyrosine hydroxylase activity in striatal synaptosomes did not show this tyrosine dependency.

PHS-6040
(Rev. 2-81)

Objectives: As the rate-limiting enzyme in catecholamine synthesis in the central nervous system, tyrosine hydroxylase is intimately involved in the regulation of neurons utilizing catecholamine neurotransmitters. In order to obtain a better understanding of *in vivo* mechanisms of regulation it is necessary to use an assay system which more closely resembles physiological conditions. Studying regulation of tyrosine hydroxylase in synaptosomes may provide more information on the effects of cofactor, ions, substrates and end-products than was previously available using a soluble assay system.

Methods: Synaptosomes were prepared from mouse whole brain and rat striatum and cortex using standard procedures. Tyrosine hydroxylase activity was quantitated by measuring formation of tritiated water after addition of ^{3}H-tyrosine. Each tube contained: synaptosomes, 150-250 μg protein; tyrosine, 2 x 10^{-7} M to 1 x 10^{-4} M; Ca^{2+}, (1 mM) or Ca^{2+}-free modified Krebs buffer. Tubes were incubated at 37°C for 10 minutes. The reaction was stopped with 0.4 ml 5% TCA. ^3H_2O was separated from remaining ^{3}H-tyrosine by a Dowex 50 x 4, activated charcoal and Dowex 1 x 2 column. ^3H_2O was quantitated by liquid scintillation spectrometry. Uptake of tyrosine and Ca^{2+} into synaptosomes was determined by standard methods. The effects of Ca^{2+} on basal catecholamine release were determined using synaptosomes preloaded with ^{3}H-dopamine (striatal) or ^{3}H-norepinephrine (whole brain, cortical).

Major Findings: Tyrosine uptake into all of the synaptosomal preparations was rapid and essentially complete after two minutes exposure to various tyrosine concentrations. Tyrosine uptake was linear over the concentration range employed

The rate of tyrosine hydroxylation in synaptosomes is dependent on the concentration of tyrosine in the external media. In whole brain and rat cortical synaptosomes the formation of DOPA is linear up to 50 μM tyrosine and begins to be saturated at 100 μM. However, in striatal synaptosomes 2 μM tyrosine appears to be saturating.

The rate of tyrosine hydroxylation can also be affected by addition of Ca^{2+} into the incubation medium. In mouse whole brain and rat cortical synaptosomes addition of Ca^{2+} increases tyrosine hydroxylase activity. The rate of tyrosine hydroxylation in striatal synaptosomes was unaffected by Ca^{2+}. This difference is not due to altered Ca^{2+} uptake since the uptake of $^{45}Ca^{2+}$ into mouse whole brain and rat striatal synaptosomes was not significantly different. Tyrosine uptake was essentially linear and similar in both rat striatal and mouse whole brain synaptosomes. In neither preparation did addition of Ca^{2+} cause an increase in tyrosine uptake. In addition it was found that catecholamine release is the same in the presence and absence of Ca^{2+} in all three synaptosomal mechanisms.

The exact mechanism of the Ca^{2+}-activation of tyrosine hydroxylase is, therefore, still unclear, however, it is possible that addition of Ca^{2+} results in activation of Ca^{2+}-activated kinases, resulting in increased phosphorylation of the enzyme.

Significance to Biomedical Research: The regulation of tyrosine hydroxylase may be important in disease states with central nervous system involvement. For example, increasing brain tyrosine concentration has been shown to lower blood pressure in spontaneously hypertensive rats. The work reported here may provide an explanation for this phenomenon. It is expected that an increased

understanding of the factors regulating tyrosine hydroxylase could result in a better understanding of how to treat disease states involving noradrenergic and dopaminergic system.

Proposed Course: Further characterization of this system will include studying tyrosine and ion interactions with tyrosine hydroxylase activity as well as cofactor concentrations, end-product inhibition and the effect of soluble protein kinases, which could result in phosphorylation of tyrosine hydroxylase.

Publications:

1. Lovenberg, W., Kuhn, D.M., Juskevich, J.: Neuronal systems and their impact on blood pressure regulation. In: Proceedings of the Innisbrook Conference - Fundamental Fault in Hypertension, Elsevier, (In press).

2. Juskevich, J., and Lovenberg, W.: Neuronal regulation of blood pressure. In: Biochemical Actions of Hormones, Vol. 8, Litwack, J., (ed.) Academic Press, New York, NY, pp. 117-165, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01865-06 HE

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Calcium-Calmodulin Dependent Activation of Tryptophan Hydroxylase by ATP and Magnesium

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

PI:	Donald Kuhn	Staff Fellow	HE	NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE	NHLBI
	James O'Callaghan	Staff Fellow, PRAT Program	HE	NHLBI
	Judith Juskevich	Staff Fellow	HE	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Tryptophan hydroxylase in rat brainstem extracts is activated 2 to 2.5 fold by ATP and Mg^{2+} in the presence of subsaturating concentrations of the pteridine cofactor 6-methyltetrahydropterins ($6MPH_4$). The activation by ATP-Mg^{2+} is also observed if the natural cofactor tetrahydrobiopterin or the synthetic cofactor dimethyltetrahydropterin is used. The activation requires ATP and Mg^{2+} and is not dependent on cyclic nucleotides. The ATP-Mg^{2+} stimulation is enhanced by calcium and can be blocked by EGTA. Removal of calmodulin from the hydroxylase containing extracts by affinity chromatography on fluphenazine-Sepharose rendered tryptophan hydroxylase unresponsive to activation by ATP-Mg^{2+}. The readdition of calmodulin restored the ATP-Mg^{2+}-induced activation in a calcium dependent manner. Drugs which bind to calmodulin also block the ATP-Mg^{2+} effect on tryptophan hydroxylase.

PHS-6040
(Rev. 2-81)

Objectives: It has been demonstrated in our laboratory (Kuhn et al., BBRC 82: 759-766, 1978) and in a number of others that tryptophan hydroxylase, the initial and rate limiting enzyme in the biosynthesis of serotonin, can be activated *in vitro* by phosphorylating conditions (ATP-Mg^{2+}). The activation of tryptophan hydroxylase is unique in that it requires calcium and shows no apparent dependence on cyclic nucleotides. The purpose of this study was to examine the role that calmodulin, the heat-stable calcium binding protein, played in the activation of tryptophan hydroxylase by ATP-Mg^{2+}.

Methods: Male Sprague-Dawley rats were decapitated and the mesencephalic tegmentum, which contains essentially all of the serotonin [5-HT] containing perikarya in this species, was rapidly dissected from the brain, frozen on solid Co_2 and stored in liquid N_2. Tryptophan hydroxylase was assayed by the method of Friedman et al. (J. Biol. Chem. 247:4165, 1972) as modified by Baumgarten et al. (J. Neurochem. 21:251, 1973). For phosphorylating conditions, the following substances were added to the incubation mixture (in the final concentrations indicated): ATP (0.5 mM) and Mg^{++} acetate (5 mM).

The fluphenazine-Sepharose affinity matrix was prepared and used according to the method of Charbonneau and Cormier (BBRC 90:1039-1047, 1979). SDS-polyacrylamide slab gel electrophoresis was carried out according to the method of O'Farrell (JBC 250:4007-4021, 1975).

Major Findings: Exposure of tryptophan hydroxylase to phosphorylating conditions (ATP-Mg^{2+}) results in a 2-2.5 fold increase in catalytic activity when the assay is carried out at subsaturating concentrations of the pteridine cofactors. Kinetic studies revealed that ATP-Mg^{2+} reduces the apparent K_m for $6MPH_4$ from 0.21 mM to 0.09 mM while having no effect on V_{max}. The apparent kinetics for the substrate tryptophan were unchanged by phosphorylating conditions.

The activation of tryptophan hydroxylase was dependent on calcium. Concentration of calcium as low as 5-10 μM stimulated the ATP-Mg^{2+} effect and EGTA blocked the activation process. Calcium could not be replaced by any other divalent cation in supporting the ATP-Mg^{2+} effect. The removal of endogenous calmodulin by chromatography of brain extracts on fluphenazine-Sepharose rendered the hydroxylase unresponsive to ATP-Mg^{2+}. The addition of purified calmodulin (1 μM) to calmodulin-free hydroxylase restored the responsiveness of the enzyme to ATP-Mg^{2+}. The restoration by calmodulin was dependent on calcium. The addition to the phosphorylation reaction mixture of drugs which are known to bind to calmodulin also block the activation of the hydroxylase by ATP-Mg^{2+}. The drugs most effective in this regard come almost exclusively from the family of agents used clinically as antipsychotics. The drugs used and the order of their inhibitory potencies are as follows: pimozide > fluphenazine > trifluoperazine > cis-flupenthizol > haloperidol > penfluridol > chlorpromazine. Other drugs such as theophylline, LSD, phentolamine, propranolol, naloxone, morphine, and BOL had no effect. The inclusion of calmodulin prevented the inhibitory effects of the antipsychotic drugs on the activation of tryptophan hydroxylase by ATP-Mg^{2+}.

Significance to Biomedical Research and Institute Programs: Phosphorylation of a protein (tryptophan hydroxylase) may play a primary role in converting 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 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.

The activation of tryptophan hydroxylase by ATP-Mg^{++} certainly suggests that a phosphorylation reaction is involved. This mechanism is novel, however, in that it is not dependent on cyclic nucleotides. Furthermore, Ca^{++} and calmodulin were found to play an important role in the activation. The present use of ligand specific affinity chromatography to identify a calmodulin dependent enzyme reaction represents the first such use of this methodology. Finally, the ability of antipsychotic drugs to inhibit the *in vitro* activation of tryptophan hydroxylase suggests that the *in vivo* mechanism of action of this class of drugs may include binding to calmodulin.

Proposed Course of Project: The following experiments are planned to more completely assess the role of calmodulin in tryptophan hydroxylation.

1. Purified calmodulin will be covalently bound to Sepharose-4B and this affinity matrix will be used to purify any protein kinases binding to the gel.

2. Rats will be treated chronically with a long acting antipsychotic, fluphenazine decanoate. At weekly intervals, groups of treated subjects will be sacrificed and the brains will be assayed in the presence and absence of phosphorylating components.

3. The effects of a variety of benzodiazepine drugs on the activation of the hydroxylase will be studied since it was recently demonstrated that purified calmodulin dependent protein kinase can be inhibited by these drugs.

Publications:

1. Kuhn, D.M., O'Callaghan, J.P., Juskevich, J., and Lovenberg, W.: Activation of brain tryptophan hydroxylase by ATP-Mg^{2+}: Dependence on calmodulin. PNAS 77:4688-4681, 1980.

2. Kuhn, D.M. and Lovenberg, W.: Calmodulin: Neurotransmitter Synthesis (Tryptophan Hydroxylase) Fed. Proc., 1981, In press.

3. Kuhn, D.M.: The regulation of tryptophan hydroxylase activity. In: Function and Regulation of Monoamine Enzymes: Basic and Clinical Aspects. E. Usdin, N. Weiner, M. Youdim (eds.), New York: MacMillan; 1981, In press.

4. Kuhn, D.M., O'Callaghan, J.P. and Lovenberg, W.: The role of calmodulin in the activation of tryptophan hydroxylase by phosphorylating conditions. Ann. N.Y. Acad. Sci. 356:399, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01867-06 HE

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

The kinetic regulation 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:	Sikta Pradhan	Guest Worker	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.8	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The goal of this work is to characterize the gluctuation in kinetic parameters of rat striatal tyrosine hydroxylase due to changes in pH and ionic concentration of the reaction mixture. As a part of this project, it is also our objective to determine the optimal conditions to observe the effect of cocaine on tyrosine hydroxylase and adenylate cyclase activity. We have also attempted to correlate changes in tyrosine hydroxylase activity with changes in adenylate cyclase activity in animals treated with cocaine.

PHS-6040
(Rev. 2-81)

Objectives: The objective of this project is to study the kinetic regulation of tyrosine hydroxylase in the rat striatum. To do this we are seeking to determine the optimum conditions under which the drug-induced changes in tyrosine hydroxylase kinetics are maximumly observed. An attempt is also made to correlate the action of cocaine on different rat brain enzymes.

Methods: Tyrosine hydroxylase is assayed using a slight modification of the tritium release method which has been described previously by Lovenberg, et al. In brief, activity is measured by determining the amount of tritiated water formed during the conversion of tritiated tyrosine to DOPA. In our assay a freshly prepared potassium phosphate-ascorbate buffer is used. This buffer is prepared by mixing 0.5 M $K_2H\ PO_4$ with 0.4 M ascorbic acid (2:1, v/v) and adjusting the pH to a value such that, after the addition of the remaining components of the assay system, the desired final pH of the reaction mixture is obtained.

The tissue extracted is purified through a Dowex (50 x 4, hydrogen form) column (0.6 x 2.0 cm) equilibrated with homogenizing buffer to remove endogenous catecholamines before the estimation of tyrosine hydroxylase activity.

Adenylate cyclase is measured by a standard enzymatic method in which cAMP is generated and subsequently assayed by radioimmunoassay.

Major Findings: Kinetic properties of tyrosine hydroxylase have been demonstrated to be markedly affected by pH of the reaction medium. As the pH of the reaction mixture is increased, a marked increase in K_m is observed at higher pH (6.0-6.6) than the optimum pH for tyrosine hydroxylase (5.8-6.0). Acute administration of cocaine increased tyrosine hydroxylase activity 10 min after injection of cocaine (20 mg/kg, i.p.) is also found to stimulate DA-sensitive adenylate cyclase located in striatal membranes, but shows no influence on the basal level of adenylate cyclase activity. The activation of DA-sensitive adenylate cyclase is maximal 5 min after cocaine injection.

Significance to Biomedical Research and Institute Program: Tyrosine hydroxylase is the rate-limiting enzyme in the catecholamine biosynthesis. Hence, considerable attention has been focused on the mechanism of kinetic regulation of this enzyme. The studies of the mechanisms by which it is regulated are potentially of great importance to the understanding of both normal and pathologic states of the central nervous system.

Proposed Course of Project: Since the current literature indicates that cAMP-dependent kinase is also involved in the regulation of tyrosine hydroxylase activity, further work should be directed towards determining the effect of cocaine (acute, subacute and chronic) on cAMP-dependent kinase and DA-sensitive adenylate cyclase and correlating them with the regulation of tyrosine hydroxylase kinetics obtained under similar conditions.

Publications:

1) Lovenberg, W., Alphs, L., Pradhan, S., Bruckwick, E. and Levine, R.: Long-term haloperidol and factors affecting the activity of striatal tyrosine hydroxylase. In: Advances in Psychopharmacology, Raven Press, N.Y., 9-15, 1980.

2) Pradhan, S., Alphs, L. and Lovenberg, W.: Characterization of haloperidol mediated effects on rat striatal tyrosine hydroxylase. Neuropharmacology, 20: 149-154, 1981.

3) Miller, L.P., Pradhan, S., Mikus, M. and Lovenberg, W.: Analysis of fluctuations in rat pineal tyrosine hydroxylase. Neurochem. Internal. (In press), 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01874-05 HE

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Adrenergic Neurons in the Brain

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

PI.	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI
OTHER:	M. Fujiwara	Visiting Fellow	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.0	PROFESSIONAL: 0.8	OTHER: 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)

Epinephrine-containing neurons are located in an area of the brain of strategic importance for the regulation of blood pressure. Although no direct rate for these cells has been established, it was of interest that while control animals possessed about 700 adrenergic calls per brain the stroke-prone SHR contained about 900 cells per brain (last year). More recently we have observed that unilateral carotid legation below the bifurcation results in a significant increase in phenylthanolamine-N-methyl transferase (PNMT) activity in the contrulateral C_1 and C_2 regions of the brainstem. Since most nerves transmitting barorecptor information cross the midline, it is concluded that the reduction in blood pressure and flow beyond the legation is causing an increase in the activity of epinephrine neurons as indexed by the enzyme PNMT. This provides circumstantial evidence that these neurons are involved in the baroreflex.

PHS-6040
(Rev. 2-81)

Objectives: The cell bodies of the epinephrine containing neurons lie in clusters in the lower brainstem. The dorsal group C_2 are very near the area postrema whereas the C_1 group lies relatively close to the ventral surface. These groups are strategically located for a role in blood pressure regulation, although no such role has been identified to date. Since it had been reported that the activity of PNMT the marker enzyme for these cells was higher in SHR than in normal rats we planned to determine whether this was accounted for by an increased number of cells. We also planned to investigate whether changes in the pattern of blood flow in the CNS by carotid artery ligation has an effect on PNMT levels.

Methods: For immunocytochemical analysis age matched stroke-prone SHR and Wistar Kyoto rats were perfused with a formaldehyde solution in buffered saline containing a 0.05% glutaraldehyde. In others experiments PNMT activity was measured by standard previously described techniques. Blood pressure was monitored by a tail cuff method.

Major Findings: A comparison of PNMT activity in the C_1 and C_2 regions indicated that the brainstem of the spontaneously hypertensive rats (SHR) contain about 25% more PNMT than control rats. This confirms previous observations in our own and other laboratories. Six hours following unilateral carotid artery ligation PNMT activity in the contralatent C_1 and C_2 region was increased 80-90%. Ipsilateral activity increased 15-20% although the apparent increase was statistically insignificant. While the baseline PNMT activity was higher in each region in the SHR, a 50-60% increase was seen on the contralateral side following carotid legation. Slight increases were observed on the ipsilateral side. Faterality of the response to carotid legation suggest that exposure of the baroreceptors to charges in blood flow and pressure result in changes in PNMT activity in the brain. This would suggest that epinephrine neurons are part of the baroreflex.

Significance to Biomedical Research and Institute Programs: It has been proposed that epinephrine neurons in the brain may be associated with mechanisms regulating blood pressure. The goal of this project is to understand how this neuronal system is regulated whether it does indeed have an impact on blood pressure.

Proposed Course: From the above studies it is clear that PNMT activity can be increased in a short period of time. Since it is unlikely to be due to new enzyme synthesis, we plan to investigate potential mechanisms for activating this enzyme.

Publications:

1) Howe, P.R.C., Lovenberg, W., and Chalmers, J.P.: Increased number of PNMT-immunofluorescent nerve cell bodies in the medulla oblongata of stroke-prone hypertensive rats. Brain Res. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01879-04 HE

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Regulation of the Ca^{2+}-Dependent Regulator (Calmodulin)

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

PI:	Ingeborg Hanbauer	Pharmacologist	HE NHLBI
OTHER:	Maurizio Memo	Guest Worker	HE NHLBI
	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

Laboratory of Preclinical Pharmacology, NIMH, St. Elizabeth's Hospital, Washington, D.C. 20032

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.5	PROFESSIONAL: 0.5	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)

Specific immunoglobulins directed toward calmodulin were produced in rabbits. An enzyme-linked immunosorbent assay was developed for the measurement of calmodulin. In rat striatum calmodulin occurs in membrane-bound and soluble form. In striatal supernatant fractions it exists in free and protein (presumably, PDE)-bound form. Hence the regulation of calmodulin was viewed in terms of biochemical processes which modulate the distribution of this protein. Chronic treatment with haloperidol increases the membrane-bound calmodulin content. Studies on the turnover-rate of this protein failed to show a change in its synthesis-rate. From these results it can be inferred that chronic treatment with haloperidol may modify the binding affinity of calmodulin to its binding site at the membrane.

PHS-6040
(Rev. 2-81)

Objectives: Calmodulin, a Ca^{2+} binding protein was first described by Cheung and subsequently studied in several laboratories. Experimental evidence indicates that calmodulin regulates the activity of a number of enzymes including adenylate cyclase, phosphodiesterase, Ca^{2+}, Mg^{2+} ATPase and protein kinase. The enzyme activation requires an initial binding of Ca^{2+} to calmodulin and this complex subsequently binds to the enzyme. The function and distribution of calmodulin indicates that in some neuronal systems it may serve as a regulatory link in coupling external stimuli to the cytoplasm in post-synaptic neurons. There exist experimental evidence that the dopaminergic system in particular is associated with calmodulin. It has been shown that in striatum and dopamine-sensitive adenylate cyclase and cAMP-PDE are regulated by calmodulin. Furthermore, during supersensitivity the calmodulin content in synaptosomal membrane is increased. In striatum the partition of calmodulin the membrane-bound and soluble pool is not yet understood, therefore this project wlll be particularly directed toward studies on the regulation of calmodulin in both cellular compartments. Studies on the turnover-rate of calmodulin will help to reveal whether during supersensitivity of dopamine-receptors the accumulation of membrane-bound calmodulin is due to enhanced synthesis rate or to a blockade of release into the cytosol.

Methods: Calmodulin measurements by enzyle-linked immunosorbent assay (ELISA). Calmodulin was purified from pig brain according to the procedure of Klee (Biochemistry 16: 1017-1024, 1977). For the production of immunoglobulins directed toward calmodulin, the purified protein was coupled to hemocyanin. This conjugate was emulsified in complete Freund's adjuvant and was injected intradermally into the back of rabbits. The injections were repeated at 2 week intervals. Partially purified immunoglobulins (50% $[NH_4]_2SO_4$ saturation) were coupled with various concentrations of calmodulin or unknown calmodulin tissue extracts. The mixtures were transferred to microplates coated with calmodulin and incubated for a period of time. Horseradish peroxidase labelled anti-rabbit globulins were added to bind on the antigen-antibody complex. Thereafter, the microplates were incubated with a O-phenylenediamine-H_2O_2 reagent and the O.D. in each well of the microplates was measured with an ELISA spectrophotometer at 488 nm.

Measurement of state of phosphoxylation of striatal membrane proteins. Striatal slices were preincubated for 30 min. After addition of dopamine receptor agonists and (or) antagonist the incubation was continued in presence of ^{32}P inorg for various periods of time. The slices were drained on filter paper and homogenized in 0.32 M sucrose and centrifuged at 1000. g. The resulting supernatant fraction was centrifuged at 100,000. g for 30 min. The resulting pellet was extracted with this buffer pH -7.4 containing either lubrol or n-octylglucosid. The extract was subjected to SDS-PAGE. The ^{32}P contained in the various proteins on the dried gel was determined by autoradiography and by liquid scintillation spectrometry.

Studies on turnover-rate of calmodulin in striatum. After chronic treatment with haloperidol a cannula was implanted into the lateral ventrical of rats. On the following day 0.50 mg cycloheximide was injected and 2 hours thereafter another 0.25 mg cycloheximide were given. At 6,8 and 12 hours thereafter the rats were killed and the calmodulin content was measured by micro-ELISA.

Major Findings; In the supernatant fraction from striatal homogenates two immunoreactive pools of calmodulin are present. Calmodulin elutes from Sephadex G-150 in two peaks. One peak elutes together with cAMP-PDE (molecular weight range of

158,000) whereas the second peak elutes together with low molecular weight proteins (25,000). The association of calmodulin with cAMP-PDE is Ca^{2+}-dependent since the PDE-calmodulin complex could be resolved by filtration on Sephadex G-150 equilibrated and eluted with 0.05 M Tris-buffer containing 1 mM EGTA. Chronic treatment with haloperidol increases the content of membrane-bound calmodulin. Measurements of turnover-rate of calmodulin show that the rate of decline of this protein is faster in saline treated rats than in haloperidol treated rats. Although the fractional rate constant was lower after haloperidol treatment, the turnover-rate was similar in both groups, because the steady-state concentration of calmodulin is higher in haloperidol treated rats. Thus, chronic treatment with haloperidol does not increase the synthesis-rate of calmodulin but may rather modify the binding affinity of calmodulin to its binding site at the membrane.

Significance to Biomedical Research and Institute Programs: The development of an enzyme-linked immunosorbent assay for calmodulin provides a new tool required to study the regulation of calmodulin content. Since the action of calmodulin has been shown to be linked to cyclic-nucleotide metabolism, studies on the partition of calmodulin between membrane and cytosol and on calmodulin-turnover rates will help to reveal some of the molecular mechanisms involved in the regulation of striatal dopamine.

Proposed Course of Project: This project will be further extended to proteins which have been shown to regulate calmodulin activity. The interaction of calcineurin and calmodulin and its physiological relevance will be included in future studies.

Publications;

1. Hanbauer, I., Pradhan, S. and Yang, H.-Y.T.: Role of calmodulin in dopaminergic transmission. Ann. N.Y. Acad. Sci., 356: 292-303, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01881-04 HE

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Effects of Pharmacological Manipulations on the Disposition of Reduced Biopterin (RT)

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

PI:	Robert Levine	Pharmacologist	HE NHLBI
OTHER:	Leonard Miller	Guest Worker	Dystonia Found.
	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.9	0.4	0.5

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Tetrahydrobiopterin (BH_4) is the rate-limiting cofactor for tyrosine and tryptophan hydroxylase, the initial enzymes which control the synthetic rates of the catecholamines and serotonin. Since aberrant metabolism of these neurotransmitters is thought to play a major role in a variety of neurological and psychiatric disorders, it was of interest to examine the disposition of BH_4 in the mammalian brain. We previously reported that a specific neurochemical lesion with 6-hydroxydopamine in the nigrostriatal system of the rat produce an 85% reduction in reduced cofactor content which suggested a high degree of localization of BH_4 to dopamine neurons in this system. We now report that total biopterin (B) and the activity of GTP-cyclohydrolase, the initial enzyme in BH_4 synthesis, are depleted approximately 70% after this lesion whereas tyrosine hydroxylase (TH) activity is reduced by 90%. Kainic acid injection in the striatum which destroys cell bodies and interneurons while sparing nerve terminals caused a 30% reduction in B and a 25% loss of TH. These results indicated that the majority of B and its biosynthetic system is localized to dopamine neurons of the nigrostriatal system. The nearly equivalent loss of B and TH after kainic acid is indicative of nonspecific aminergic terminal damage.

PHS-6040
(Rev. 2-81)

Objectives: In order to more fully understand the consequences of therapeutically manipulating rates of biogenic amine synthesis by altering BH_4 levels in the central nervous system, it is important to determine the various systems in which BH_4 may have a physiological role. Through the use of specific neurochemical lesions, we have atttempted to gain a further understanding of the relationship between BH_4 and central aminergic neurons as well as to investigate the likelihood of alternative roles for BH_4 in cellular function. Answers to these questions will provide useful information regarding the potential therapeutic administration of BH_4 in certain neurological and psychiatric disorders. Our studies may also unveil novel concepts regarding the nature of the interactions between BH_4 and the aromatic amino acid hydroxylase enzymes, specifically tyrosine hydroxylase.

Methods: Male Sprague-Dawley rats (250-300 g) were used for all 6-hydroxydopamine (6-OHDA) and kainic acid (KA) lesion experiments. Animals were anesthetized with chloropent (2.2 ml/kg) and placed in a David Kofp stereotaxic instrument. In one set of animals, 6-OHDA was injected (8 μg/4 μl) in the left substantia nigra (SN) to destroy the nigrostrictal dopaminergic neurons. The coordinates from bregma used for injection of 6-OHDA in the SN were as follows: anterior-posterior (AP) = -5.2 mm, medial-lateral (ML) = + 2.0 mm, dorsal-ventral (DV) = -7.1 mm (from cortical surface). Two weeks after the operation, the effectiveness of the lesion was examined by monitoring apomorphine-induced rotational behavior. After apormorphine injection, animals were placed in a round bowl and turns were monitored by an observer for thirty minutes after an initial five minute post-injection interval during which turning was not quantitated. Greater than three clockwise turns/minute for a thirty minute period constituted a successful lesion. Only positive turning animals were used for subsequent biochemical assays described below. 6-OHDA treated animals were sacrificed two weeks after testing rotational behavior.

In a separate group of animals, KA was injected (2 μg/1 μl) unilaterally in the left striatum to destroy striatal cell bodies and interneurons while causing minimal damage to striatal nerve terminals. The coordinates from bregma used for KA injection in the striatum were as follows: AP = 0.0 mm, ML = +3.0 mm, DV = -4.0 mm (from cortical surface). The success of KA lesions was determined by measuring substance P-like immunoreactivity (SPLI) in both the anterior striatum (substance P cell body location) and SN (SP terminal area). These structures comprise the striatonigral SP pathway which is reciprocal to the nigrostriatal dopamine pathway. KA-treated animals were sacrificed 22 days after the lesion.

Control and lesioned animals were sacrificed by decapitation, brains were quickly removed and the appropriate areas were dissected from coronal slices which were obtained by using single-edged razor blades placed in a slotted plexiglass brain block which was maintained at $4^{\circ}C$ on ice. All dissected tissues were frozen in liquid nitrogen until time of assay.

Substance P-like immunoreactivity was measured in the substantia nigra and striatum by radioimmunoassay which was previously developed in our laboratory. Reduced pterin cofactor content in certain experiments was measured in the nigra and striatum by the purified phenylalanine hydroxylase assay with minor modifications as described in detail in last year's annual report. For striatal samples in which tyrosine hydroxylase, biopterin, and GTP-cyclohydrolase were to be simultaneously assayed, a novel homogenization protocol was employed.

Striated samples weighing between 20 and 40 mg were homogenized in 450 µl of ice-cold .05 M potassium phosphate buffer, pH 6.0. The homogenate was rapidly divided into four parts (using an SMI pipet) to allow the measurement of GTP-cyclohydrolase activity (150 µl homogenate), botal biopterin (75 µl), oxidized cyclohydrolase activity (150 µl homogenate), botal biopterin (75 µl), oxidized biopterin (75 µl), and tyrosine hydroxylase activity (approximately 150 µl) from the same tissue sample. To the tubes containing hemogenate for biopterin determination, 25 µl of 0.4N H_3PO_4 was added immediately (which were then frozen on dry ice) to provide an acid environment and ensure stabilization of the cofactor.

The more specific high pressure liquid chromatography (HPLC) assay recently described by Fukushima and Nixon (1980) was used to measure oxidized and total striatal biopterin content. A Varion LC 5000, a Waters reverse-phase (C-18) column, and a Perkin Elmer 650-10S flourescence spectrophotometer were used to separate and quantitate the native flourescence of biopterin (excitation:350 nm, emission:445 nm). A mobile phase of 5% methanol-water was used to elute off the HPLC the biopterin which was generqated from tissue by the differential iodine oxidation procedure. Essentially, iodine oxidaion of the striatal homogenate under acid conditions, which yields a value of the total biopterin pool, converts all oxidative forms of the cofactor to biopterin. This includes, tetrahydrobiopterin (BH_4), quinoid-dihydrobiopterin (q-BH_2), dihydrobiopterin (BH_2), and biopterin (B). Conversely, under basic conditions, only the oxidized forms of biopterin (BH_2 and B) are detectable as biopterin on the HPLC. The quantity of biopterin detected by acid iodine oxidation minus the quantity of biopterin detected by basic iodine oxidation yields the value of the reduced biopterin pool (BH_4 plus q-BH_2) in the striatum. Striatal homogenates were differentially oxidized for one hour at room temperature under reduced light, centrifuged at 39,000 x g for 15 min at 4^oC, and the supernatants applied to a 6 x 10 mm Dowex-50 x 8 (H^+, 200-400 mesh) cation exchange column. Columns were washed with 6.0 ml of H_2O and eluted with 6.0 ml of 0.25 N ammonium hydroxide directly onto a 6 x 5 mm Dowex 1-X8 (100-200 mesh). These columns were washed with 4.0 ml H_2O and eluted with 2.0 ml 1.0 N HCl. Eluates were frozen and lyophilized to concentrate the sample. Samples were redissolved in 150 µl H_2O and 50 µl were injected on to the HPLC. Neopterin was used as an internal standard since no neopterin can be detected in rat tissues. Striatal biopterin content was quantitated by biopterin/neopterin-varied biopterin additions to control striatal homogenates. GTP-cyclohydrolase activity was determined by measuring the amount of product, dihydroneopterin triphosphate, formed from GTP. After centrifugation of the striatal homogenates at 39,000 x g for 15 min at 4^oC, a 125 µl aliquot of each supernatant was passed over separate 5 mm x 24 cm columns of Sephadex G-25 preequilibrated with Tris-HCl, pH 8.2. The column was rinsed with 50 mm Tris-HCl, pH 8.2 and the majority of the eluate protein was collected in a 700 µl fraction. For determination of enzyme activity, 450 µl of this fraction was incubated at 42^oC for 2 hr with 50 µl of Tris buffer containing GTP in a final concentration of 400 µM. The reaction was terminated by the addition of 80 µl 2 N TCA. To convert from reduced to the more stable oxidized form of neopterin triphosphate, the samples were kept at room temperature under reduced light for 30 min following the addition of 100 µl 0.2N TCA containing 0.5% I_2/1% KI. The unreacted iodine was reduced with 40 µl of 1% ascorbic acid and pH reestablished to 8.2 with 2N NaOH. The neopterin triphosphate was then converted to neopterin by incubating with calf intestine alkaline phosphatase (10 units) at 37^oC for 1 hr. This reaction was then terminated with 80 µl 2N TCA. Neopterin was then isolated and quantitated by the same methods as used for biopterin which were described above. However, biopterin was

used as an internal standard while neopterin was varied in striatal supernatants which were incubated in the absence of GTP. To the lesioned and control stratal tissues, standard biopterin was added after the alkaline phosphatase reaction. Linearity of enzyme activity with regard to time and protein concentration in the strictum was confirmed.

Major Findings: We reported last year that a 6-OHDA lesion of the SN depleted reduced cofactor content by 85% in both the SN and striatum. Through a more complete analysis of the effects of this 6-OHDA lesion, we have demonstrated that biopterin (B) content and the activity of GTP-cyclohydrolase (CH), the initial enzyme in BH_4 synthesis, are depleted by approximately 70%, whereas tyrosine hydroxylase (TH) is diminished by 93%. These results suggest that the majority of B and its biosynthetic system are highly co-localized in the dopamine neurons of the nigrostriatal system. The difference between the degree of depletion of TH(93%) and B and CH (both 70%) probably represents the cofactor synthetic system which is required for the activity of tryptophan hydroxylase and contained within the serotonergic nerve terminals innervating this region. Thus, it appears that within the nigrostriatal system, B and CH are exclusively associated with aminergic neurons. If one can extrapolate this relationship to other areas of the brain, it is probable that within the brain, the exclusive physiologic role for BH_4 is to serve as the hydroxylase cofactor for the aromatic amino acid hydroxylase enzymes.

The kainic acid (KA) injections in S reinforce this concept. KA caused a 25% loss in striatal TH activity and a 30% loss in striatal B content. Since TH is exclusively contained within catecholaminergic neurons (dopamine neurons in the nigrostriatal system), the nearly equivalent loss of TH and B from the striatum indicate that KA has some nonspecific dopamine nerve terminal damage. These results support the idea that B is exclusively synthesized and utilized by aminergic neurons in brain.

Having established the predominant localization of BH_4 to dopamine neurons in the nigrostriatal system, one can calculate the molar ratios and concentrations of BH_4 and TH. On a gross wet weight basis assuming a ubiquitous distribution of BH_4 in S, the concentration of BH_4 is approximately 1 μM. However, we know now that BH_4 is highly concentrated in the dopamine nerve terminals which constitute a relatively small percentage of the total volume of the striatum. If we assume this percentage to be 1% of the total striatal volume, then the concentration of BH_4 would be 100 μM, which is much higher than was previously thought. Surprisingly, if one calculates the concentration of TH molecules in the striatal dopamine terminals, the TH concentration is also approximately 100 μM. Therefore, it appears that TH and BH_4 coexist in roughly a 1:1 molar ratio and are much more highly concentrated than previously thought. These observations open up many interesting avenues for future research.

Significance to Biomedical Research and Institute Programs: These studies have contributed further to our understanding of the relationship between tetrahydrobiopterin and aminergic neuronal systems in brain. The predominant localization of BH_4 to dopamine neurons of the negrostriatal system has led to our reevaluation of the relationships between BH_4 and tyrosine hydroxylase with these neurons. The fact that BH_4 appears to be a physiological cofactor only in aminergic neurons has clinical implications. Our attempts (in collaboration with NINCDS) to elevate

brain BH_4 levels and ameliorate the clinical symptoms in patients with Parkinson's disease should be aided because the probability of therapeutically administered BH_4 influencing other physiological systems may be lessened. Therefore we may be able to specifically target BH_4 to elevate aromatic amino acid hydroxylation and thus enhance neurotransmitter synthesis in aminergic neurons, particularly in the surviving dopamine neurons of the nigrostriatal system.

Proposed Course of Project: There are aspects of the catecholamine generating system in brain which have not been examined in the same manner as BH_4 or GTP-cyclohydrolase. For instance, there is some controversy as to the degree of association of quinoid dihydropterin reductase (QDPR) with aminergic neurons in brain. Since QDPR is ultimately responsible for the regeneration of BH_4 from quinoid-BH_2 our lesion protocol will be used to determine the degree of association of QDPR with aminergic neurons in brain.

To provide information which may be necessary for the collaborative clinical study, the time course of BH_4 entry into the CNS will be determined after I.P. administration. To determine if BH_4 levels are capable of elevating dopamine synthesis in the striatum, BH_4 will be administered and the major metabolites of dopamine (DOPAC and HVA) will be monitored as an index of turnover of this neurotransmitter.

Publications:

1. Lovenberg, W., Levine, R.A., and Miller,L.P. (1981); The Hydroxylase Cofactor and Catecholamine Synthesis. In: Second Conference on Monoamine Enzymes; Macmillan Publishers Ltd. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01884-03 HE

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Enkephalin-like Peptides and Cardiovascular Control

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

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OTHER:	G.D. Kelly	Biological Lab. Tech.	HE NHLBI
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LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.4	PROFESSIONAL: 0.4	OTHER:

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
Secretion of enkephalin-like peptides immunoreactive to (met^5) or (leu^5-enkephaline from adrenal glands into circulation was studied in dogs with indwelling cannulae in the lumbar adrenal vein. The stimulation of the splanchnic nerve causes a voltage-dependent increase of met-enkephaline immunoreactive peptides in adrenal venous plasma. The peptide secreted from adrenal gland was identified to be met-enkephalin by Bio-Gel P-2 chromatography followed by high performance liquid chromatography. Injection of morphine also increased the plasma content of these peptides which was blocked by naloxone and hexamethonium. The effect of splanchnic nerve stimulation was mimicked by dimethylphenylpiperazinium and could be blocked by hexamethonium. This study clearly indicates that the release of met-enkephalin-like peptides from adrenal glands is mediated through activation of nicotinic receptors.

PHS-6040
(Rev. 2-81)

Objectives: Recent reports suggest that met-enkephalin-like peptides may function as neurotransmitters or neuromodulators in sympathetic ganglia (DiGuilio, A.M. et al., Neuropharmacol. 17:989-992, 1978) and adrenal medulla (Yang, H.-Y.T. et al., Fed. Proc.; Schultzberg, N. et al., Neurosci. 3:1169-1186, 1978). These met-enkephalin-like peptides are concentrated in adrenal chromaffin granules, although the splanchmic nerve innervating the cortex and medulla of the adrenal gland also contain these peptides. Studies on primary cultures of bovine chromaffine cells showed that met-enkephalin-like peptides can be released by nicotinic receptor stimulation (Kumakura et al., Nature 283:489-492, 1980. When released from afferent axons, they may act on opiate receptors located in peripheral nervous tissue.

The principal effect of narcotics upon the circulation seems to be hypotension. This is mainly caused by vasodilation and peripheral pooling of blood, although the mechanism involved is vaguely understood. The vasodilatory hormones were to be secreted from sympathetic nerves or adrenal chromaffin granules, and might cause vasodilatation by acting on distant receptors.

In view of the possible role of met-enkephalin-like peptides in the regulation of sympathetic transmission and vascular tone, we have studied the content of met-enkephalin-like peptides in adrenal gland and in the adrenal venous outflow after electrical stimulation of the splanchnic nerve or direct stimulation of nicotinic or muscarinic receptors in adrenal chromaffin cells.

Methods: Collection of adrenal venous blood. Female American foxhounds (1 year old) were fitted with an indwelling catheter in the left lumbar adrenal vein. The sampling of adrenal venous blood was carried out both in conscious and anesthetized dogs. In some animals the splanchnic nerve was transected at least 3 days prior to the experiment.

Blood pressure measurements. Dogs were anesthetized with pentobarbital and cannulae were implanted into the femoral vein and femoral artery. The mean arterial blood pressure was recorded from normal and reserpinized dogs before and during electrical stimulation of the splanchnic nerve.

Measurement of met-enkephalin-like peptides in adrenal venous plasma. Enkephaline-like peptides were extracted from plasma by acetone precipitation in acid, medium (0.01 N HCl). The extract was centrifuged and the supernatant was extracted 4 times with petroleum ether (4°C), (Acetone extract: Petroleum ether = 1:1). Residual acetone and petroleum ether were removed by a stream of N_2 and the remaining aqueous solution was lyophilized. The residue was redissolved in H_2O, heated at 100°C for 10 min and centrifuged at 12,000 x g for 10 min. Aliquots of the supernatant were assayed radioimmunochemically using an antibody raised against met-enkephalin.

Identification of met-enkephelin-like peptides. The plasma was extracted as described above, lyophilized and redissolved in 1N acetic acid. The solution was applied on Bio Gel P2 column and eluted with 1N acetic acid. Fractions containing met-enkephaline-like peptides were pooled, lyophilized and reconstituted in 1M pyridine/0.5 M acetic acid and pumped onto a HPLC reverse Bio-Sil ODS10 column. The peptides were eluted from the column with a gradient of 1-propanol in 1N pyridine/0.5 M acetic acid.

Major Findings: Secretion of met-enkephalin-like peptides from adrenal glands elicited by splanchnic nerve stimulation. The content of met-enkephaline-like peptides was highest in adrenal venous plasma (0.087 pmol/ml plasma), whereas plasma sampled from jugular vein, femoral vein and carotid artery contained less (0.045 pmol/ml plasma). In anesthetized dogs electrical stimulation of the splanchnic nerve with increasing voltage from 5 to 30 V results in a voltage-dependent release of (met^5)-enkephalin-like peptides into the adrenal venous blood ranging from 150 to 300 % over control. BioGel P-2 filtration and HPLC chromatography of plasma extracts before and after stimulation revealed the presence of both high and low molecular weight peptides and showed that splanchnic nerve stimulation increases the plasma content of the low molecular weight species.

Measurement of the mean arterial blood pressure during splanchnic nerve stimulation resulted in an increase above control. However, if the dog received 3 injections of reserpine (0.5 mg/kg daily) which impairs the storage of catecholamines in the adrenal gland, stimulation of the splanchnic nerve caused a decrease of the mean arterial blood pressure.

Secretion of (met^5)-enkephaline-like peptides from adrenal glands elicited by morphine injection. Injection of morphine increases the amount of (met^5)-enkephalin-like peptides released into the adrenal venous plasma. Pretreatment of dogs with naloxone hexamethonium or prior transection of the splanchnic nerve were able to prevent this increase.

Significance to Biomedical Research and Institute Programs: Enkephalin-like peptides have been studied more extensively on their control of pain threshold, but the physiological function of these peptides does not appear to be limited to this effect. Since narcotic agents of clinical values are always showing respiratory and vasodepressant effects and since the injection of morphine increases the content of (met^5)-enkephalin-like peptides in venous blood it appears important to reveal the action mechanism of these peptides on the cardiorespiratory system.

Future experiments are planned to improve the accurate quantitation and the determination of the molecular nature of the enkephaline-like peptides secreted from adrenal glands. The physiological role of these peptides which are released together with catecholamines has to be defined. In the view that enkephaline-like peptides may act as neuromodulators, we will study the role of these peptides in the neuronal modulation of cardio-vascular circulation.

Publications:

1. Hexum, T.D., Hanbauer, I., Govoni, S., Yang, H.-Y.T. and Costa, E.: Secretion of enkephaline-like peptides from canine adrenal gland following splanchnic nerve stimulation. Neuropeptides 1:137-143, 1980.

2. Costa, E., Guidotti, A., Hanbauer, I., Hexum, T.D., Saiani, L. and Yang, H.-Y.T.: Regulation of cholinergic transmission in adrenal medulla, In: Proceedings of the International Symposium on Cholinergic Mechanisms (eds., G.C. Pepeu and H. Ladinsky), Plenum Press, New York (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01885-03 HE

PERIOD COVERED
October 30, 1980 through September 1, 1981

TITLE OF PROJECT (80 characters or less)

Development of chromatographic techniques for study of substance P in tissue samples. (Revised Title)

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

PI:	Frank Douglas	Clinical Associate	HE	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

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)

An attempt was made to apply chromatographic techniques, particularly affinity column and high pressure liquid chromatography, to the identification and quantification of substance P in rat brain tissue. The affinity column was made from monoclonal antibody (Ref. 1) linked to CN sepharose 4B. The affinity column was compared to Sep Pak C_{18} and Silica Sep Pak and found to be superior to both in separating substance P-like immunoreactive peptides from other peptides contained in substantra nigra and hypothalamus of rat brain. An added feature is that the mobile phase of acetomibile/water/phosphoric acid/trithylamine in ratio 28% of: 72%: 0.1% to 0.025% (v/v) used in the HPLC is also excellent for eluting substance P-like peptides that are bound to the antibody in the affinity column. The HPLC method described has a sensitivity of 5-10 µg.

PHS-6040
(Rev. 2-81)

Objectives:

1) To develop an HPLC method with sensitivity in the 0.1-1.0 μg range for measurement of substance P.

2) To develop techniques for pretreatment of rat brain tissue to concentrate substance P-like immunoreactive peptides prior to isolation by HPLC.

Methods: Substantra Nigra and hypothalamus were dissected at 4°C immediately after decapitation of 200-250 g Sprague-Dawley rats. Tissue from 6-10 animals were pooled, homogenized in 0.01 N HCL and heated in boiling water for 10 mins. The homogenalis were centrifuged at 3000 rpm for 20 mins. The supernatant was decanted, lyophilized, and subsequently dissolved in appropriate solvents.

The HPLC method consisted of use of Waters 6500 pump, WISP antomatic injector, and DuPont TMS column and UV spectropholinelis. Mobile phase consisted of 28% acelbuitrile, 0.1% triflurroacetic acid or phosphoric acid and 0.025% triethylamine.

The affinity column was prepared by linking monoclonal SP antibody to activated CN Br-Sepharose. The method outlined by Pharmacia in the product manual was used.

Results: Elution profiles of I^{125} tyrosine substance P and substance P of C_{18} Sep Pak and affinity column compared with Silica Sep Pak. 80% of the sample was recovered in the first 2 ml of wash on the affinity column compared with 65% for C_{18} Sep Pak and 35% for Slica Sep Pak. The recovery of SP from rat hypothalmus or substantia nigra was 50% with the affinity column and 30% with the C_{18} Sep Pak. Recovery with Silica Sep Pak was less than 15%. In addition, the HPLC chromatograms of brain tissue were cleaner when pre-prepared with affinity column 18 on with Sep Pak.

Significance to Biomedical Research and Institute Programs: The affinity column presents the possibility of selectively concentrating substance P-like immunoreactive peptides from brain tissue. This can be coupled with the HPLC for detecting and in the future, measuring SPLI in tissue.

Publications: In preparation: Use of affinity column in detection of substance P-like immunoreactivity in rat brain.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03501-03 HE

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Effects of Morphine on Calcium-Regulated Phosphorylation (Revised Title)

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

PI:	Linda Kennedy	Guest Worker	HE NHLBI
OTHER:	Judith Juskevich	Staff Fellow	HE NHLBI
	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 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)

The administration of morphine in vivo (3-30 mg/kg) produced a dose-dependent increase in the calcium-dependent phosphorylation of three synaptosomal cytosolic proteins having molecular weights of 50,000, 55,000 and 60,000. This effect, which could be abolished by pre-treatment with naloxone, was also observed following the administration of levorphanol and l-methadone. The analgesically inactive stereoisomers, dextrorphan and d-methadone, were ineffective. In rats treated chronically with morphine, phosphorylation patterns were similar to those observed in placebo-treated controls. Morphine was also less effective in stimulating phosphorylation when administered acutely as a challenge dose to chronically-treated animals.

PHS-6040
(Rev. 2-81)

Objectives: A number of recent studies have demonstrated neurochemical changes in the central nervous system following acute and chronic administration of morphine. In particular, morphine has been reported to alter the activity of adenylate cyclase, as well as the levels of cAMP, in several brain regions. Morphine also alters both the levels and binding of calcium in brain. Since both cAMP and calcium are known to stimulate protein phosphorylation in synaptic membranes and synaptosomal cytosol, morphine administration might also influence protein phosphorylating systems in brain. Such systems, which include the enzymes protein kinase and phosphoprotein phosphatase, are probable links in the series of biochemical signals linking receptor stimulation and physiological effects, and thus represent a locus at which the effects of endogenous and exogenous opiates might be mediated. The objective of the present investigation was therefore to examine the effects of acute and chronic administration of morphine and related drugs on calcium and cAMP-dependent phosphorylation of cytosolic proteins from rat striatal synaptosomes.

Methods: Tissue preparation. Male, Wistar rats were killed by decapitation, the brains removed and the striata dissected within 30 seconds. A crude synaptosomal lysate was prepared from a striatal homogenate by differential centrifugation. This lysate was centrifuged at 140,000 x g for 90 minutes. The resulting supernatant fraction was used as a source of synaptosomal cytosol.

Phosphorylation assays. The net incorporation of phosphate into striatal synaptosomal cytosolic proteins was assayed at 30°C in a standard assay mixture containing (200 μl final volume) 50 mM HEPES, pH 7.0, 10 mM $MgCl_2$, 1.0 mM DTT and 5 μM (γ ^{32}P) ATP. Following a one minute preincubation of the assay mixture, the phosphorylation reaction was initiated by the addition of the cytosol. After one minute the reaction was terminated by solubilizing the cytosolic proteins in 100 μl of a sample buffer containing 9% SDS, 0.03 M Tris-HCl buffer, pH 8.0, containing 6% mercaptoethanol, 3 mM EDTA and 27% sucrose. All samples were assayed at least in duplicate.

Gel Electrophoresis, Autoradiography and Microdensitometry. Proteins were resolved on SDS polyacrylamide slab gels. The acrylamide concentration was 6% and 10% in the stacking and resolving gels, respectively. Both stacking and resolving gels contained SDS at a final concentration of 0.1%. Following electrophoresis the gels were fixed and stained with 0.1% Coomassie blue R250 and 50% methanol, 10% acetic acid before drying under heat and vacuum. Autoradiography was carried out for a period of 1-2 days using Kodak RP X-ray film. The molecular weights of the protein bands were estimated from molecular weight standards. The incorporation of phosphate into specific protein bands was determined by microdensitometry of the autoradiographs using and EC 920 transmission microdensitometer. Protein phosphorylation was quantified from the densitometric scans by integration of the areas under the peaks corresponding to specific protein bands.

Major Findings. In vitro, morphine (.001-1 mM) had no effect on the phosphorylation of cytosolic proteins prepared from rat striatal synaptosomes. In contrast, in vivo administration of morphine (3-30 mg/kg) 30 minutes prior to sacrifice resulted in a maximal increase of 200% in calcium-dependent phosphorylation of synaptosomal cytosolic proteins with molecular weights of 50,000, 55,000 and 60,000. This effect, measured in the presence of 50 μM Ca^{++}, was

not cAMP-dependent, and was not observed in synaptosomal membrane fractions. Effects on phosphorylation similar to those observed following morphine administration were also produced by treatment with levorphanol (2 mg/kg) or l-methadone (5 mg/kg), although the analgesically inactive isomers dextrorphan and d-methadone were ineffective. Moreover, the effect of morphine could be blocked by pretreatment with the narcotic antagonist naloxone (2 mg/kg).

In a related series of experiments, the effects of chronic morphine administration on calcium-dependent phosphorylation of cytosolic proteins were examined. In these studies, rats were implanted subcutaneously in the nape of the neck with morphine pellets (75 mg) 72 hours prior to sacrifice. Phosphorylation patterns observed in chronically-treated rats were similar to those observed in placebo-implanted controls, implying that tolerance to this neurochemical effect of morphine had developed within 72 hours of continuous exposure to morphine. Furthermore, morphine was much less effective in stimulating calcium-induced phosphorylation when administered acutely as a challenge dose (75 mg/kg) to chronically-treated rats.

Significance to Biomedical Research and Institute Program: Although an involvement of opiate action in the dynamics of several neurotransmitter systems has been demonstrated, little progress has been made toward a delineation of the specific molecular mechanism(s) involved in the acute and chronic effects of narcotic analgesics.

The studies described above demonstrate effects of acutely-administered morphine on a calcium-regulated phosphorylation system in synaptosomal cytosol, and further reveal that tolerance to these effects occur if animals are chronically treated with morphine. Thus, calcium-regulated protein phosphorylation represents one possible locus of opiate action that may account for some of the acute and chronic effects of these drugs. Furthermore, calcium sensitive phosphorylation systems may be of physiological as well as pharmacological importance since the effects of the endogenous opiates, the endorphins, may also be mediated through an effect on calcium-regulated protein phosphorylation.

Proposed Course of Project: The effects of calcium ion on cytosolic protein phosphorylation may in some cases be regulated via the calcium binding protein calmodulin. Calmodulin distribution in striatal tissue slices is altered by incubation of the slices in the presence of morphine, such that the content of calmodulin in the cytosol, the site of morphine-induced changes in calcium-dependent phosphorylation, increases. These observations, taken together, suggest a possible relationship between calcium availability, calmodulin, and protein phosphorylation in mediating the effects of morphine. Future experiments will therefore be designed to examine the effects of acute and chronic morphine treatment on calmodulin levels and subcellular distribution in rat striatum, and to relate changes in this parameter to changes in protein phosphorylation patterns. In addition, the effects of morphine on other neurochemical indices of receptor stimulation, such as adenylate cyclase activity and receptor sensitivity, will be studied in an attempt to delineate the series of biochemical events which mediate the observed effects on phosphorylation.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03503-03 HE

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Characterization of Proenzyme I from Rat Brain (Revised Title)

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

PI:	Vivian S. Zabrenetzky	NIH Postdoctoral Fellow	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI
	Donald M. Kuhn	Staff Fellow	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.0	.2

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A trypsin-sensitive, cyclic nucleotide independent prokinase from rat brain supernatant contains a calmodulin-like subunit. It phosphorylates histone type II in a Ca^{2+}-dependent manner, and histone type H_1 in a phospholipid dependent manner. It may phosphorylate tryptophan hydroxylase in a calmodulin dependent manner. It may phosphorylate synaptic plasma membrane in a calcium and calmodulin-dependent manner as well.

PHS-6040
(Rev. 2-81)

Objectives: Prokinase C contains calmodulin as a subunit. Tryptophan hydroxylase is activated by phosphorylation in a calcium-calmodulin dependent manner, therefore the role of the kinase in activation of the hydroxylase will be determined. Since synaptic plasma membranes contain proteins that are phosphorylated in a calcium-calmodulin dependent manner, it will be determined whether prokinase can utilize these proteins as endogenous substrates.

Methods: The enzyme is purified through DEAE, Sephadex G-100 chromatography and isoelectrofocusing. Polyacrylamide slab gel electrophoresis (PAGE) is used to determine the subunit structure of the enzyme. Fluphenazine and fluphenazine-Sepharose affinity chromatography are used to determine the presence of calmodulin as a subunit of the enzyme.

Major Findings: Highly purified prokinase C was obtained by the isolation procedure described above. Three min. incubation with trypsin caused at least a 10 fold activation of the kinase. Either before or after proteolytic activation, the enzyme was essentially not stimulated by cyclic nucleotides (cAMP or cGMP). It showed optimal activity towards histone type II as substrate at the non-physiological level of 75 mM Mg^{2+}. Purified enzyme which had been dialyzed against .025 M tris (pH 7.5) was free of Ca^{2+} and showed a two-fold enhancement of activity toward histone type II after addition of 40 μM $CaCl_2$, at 4mM Mg^{2+}. The Ca^{2+} dependency requires the presence of a high concentration of 2-mercaptoethanol (30 mM). At 10 mM 2-mercaptoethanol there is inhibition of activity at higher Ca^{2+} concentrations. This rules out the presence of a contaminating Ca^{2+}-dependent protease as the cause of activation. This was confirmed by the lack of effect on Ca^{2+} stimulation by phenylmethylsulfonyl fluoride and leupeptin which are protease inhibitors.

The prokinase also showed Ca^{2+} dependency in the presence of phospholipid/neutral lipid (phosphatidyl inositol/diolein), with histone H_1 as preferred substrate. This mode of Ca^{2+} stimulation occurred in the absence of 2-mercaptoethanol (5 fold stimulation) but was enhanced further (8 fold) by the presence of 2-mercaptoethanol (30%).

Ca^{2+} stimulation in the presence of 30 mM 2-mercaptoethanol and in the absence of phospholipid was blocked by the addition of the calmodulin inhibitor-fluphenazine. Fluphenazine-linked Sepharose has a specific affinity for calmodulin in the presence of Ca^{2}. A fraction of the purified enzyme was retained on the affinity column in the presence of Ca^{2+} and was released by EGTA. This fraction appeared to contain a calmodulin-like subunit as determined by SDS-PAGE in 10% slab gels. The EGTA fraction was trypsin sensitive.

Judith Juskevich has found that rat brain synaptic plasma membranes contain proteins that are phosphorylated by a Ca^{2+}-calmodulin dependent kinase. Preliminary experiments show that some of these proteins are phosphorylated by purified prokinase C.

Significance to Biomedical Research and Institute Programs: Prokinase C contains calmodulin as a subunit and is therefore only one of three kinases (myosin light chain kinase, and phosphorylase kinase) to have such a subunit. It has long been established that calcium modulates neuronal transmission and that phosphorylation mediates this process. Since the prokinase contains calmodulin it could be a link between the two processes. It may provide this link by activating

tryptophan hydroxylase and thereby regulating serotonin synthesis in a calcium-dependent manner. Or, it may phosphorylate synaptic membrane proteins to maintain the proper homeostatic level of membrane phosphorylation in preparation for synaptic transmission. The mechanism of action of these two processes could be elucidated by studying the effects of prokinase on them.

Proposed Course of Project: The activity of the enzyme as a modulator of catecholamine synthesis is being studied by the addition of tryptophan hydroxylase to the purified enzyme under phosphorylating conditions. The activation of the enzyme will be determined with special reference to its calcium dependent action.

The ability of the prokinase to phosphorylate synaptic plasma membranes as endogenous substrates, in a calcium and calmodulin-dependent fashion will be determined.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03505-03 HE

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Effects of Pteridine Cofactors on the Stability of Tryptophan Hydroxylase and Tryosine Hydroxylase (RT)

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

PI:	Donald M. Kuhn	Staff Fellow	HE	NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE	NHLBI
	Belle Ruskin	Chemist	HE	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 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)

The stability of tryptophan and tyrosine hydroxylases was studied in vitro. The activity of tryptophan hydroxylase decays rapidly when preincubated at 37°C for up to 60 min. The inclusion of tetrahydrobiopterin (BH_4), 6-methytetrahydropterin ($6MPH_4$), or dimethyltetrahydropterin ($DMPH_4$) in the preincubation mixture significantly protects tryptophan hydroxylase from O_2-thermal inactivation. The protective effects were provided only be reduced cofactor since biopterin did not present inactivation. Tyrosine hydroxylase, on the other hand, is stable throughout a 60 min preincubation and the inclusion of BH_4, $6MPH_4$, or $DMPH_4$ in the preincubation causes a loss of catalytic activity. The inhibitory substance is not a peroxide or superoxide anion, nor is it the cofactor or catecholamine end-product.

PHS-6040
(Rev. 2-81)

Objectives: Tryptophan and tyrosine hydroxylase are the initial and rate limiting enzymes in the synthesis of serotonin and catecholamines, respectively. Purification and characterization of these important enzymes has been hindered as a result of their instability, especially tryptophan hydroxylase (Kuhn et al., JBC 255:4137-4143, 1980). Since it is a general phenomenon that many enzymes are more stable in vitro in the presence than in the absence of their substrates, we investigated whether the pteridine cofactors (cosubstrates) would alter the stability of either tryptophan of tyrosine hydroxylase.

Methods: Tryptophan hydroxylase was assayed in rat mesencephalic tegmental extracts by the method of Baumgarten et al (J. Neurochem. 21:251, 1973). Tyrosine hydroxylase was assayed in rat striatal extracts by the method of Lerner et al. (Neurochem. Res. 3:641, 1978). For stability studies, enzyme containing extracts were incubated at 37°C in room air for 0-60 min in the presence or absence of BH_4, $6MPH_4$, or $DMPH_4$. Aliquots were removed at 15 min intervals for the appropriate enzyme assay.

Major Findings: Preincubation of tryptophan hydroxylase at 37°C for varying periods of time leads to a loss of catalytic activity. After 30 min of preincubation, activity is reduced to approximately 50% of starting activity. The addition of BH_4 (0.25 mM), $6MPH_4$ (1.0 mM), or $DMPH_4$ (0.5 mM) to the preincubation mixture protects tryptophan hydroxylase from inactivation. To exert protective effects the pteridine cofactors must be chemically reduced since the oxidized form of BH_4, biopterin, is without effect. Tyrosine hydroxylase, on the other hand, is quite stable throughout the entire preincubation period. After 60 min preincubation there is no more than a 10% loss of catalytic activity. The addition of BH_4, $6MPH_4$, or $DMPH_4$ surprisingly caused a rapid loss of tyrosine hydroxylase activity. As before, the inhibitory effects of the pteridines was dependent on the reduced forms since biopterin did not cause a loss of activity.

It is known that catecholamines exert end-product inhibition on tyrosine hydroxylase and it is possible that some catecholamines were synthesized during preincubation. Extracts were chromatographed on ion exchange (Dowex 50) columns to remove catacholamines after 60 min of preincubation, and after this step, the tyrosine hydroxylase remained inhibited. Since the Dowex 50 would also remove BH_4, the pteridines as well as the catecholamines can be ruled out as the inhibitory agents. Incubation of striatal extracts (in the presence of BH_4) with catalase or superoxide dismutase did not present the loss of activity excluding peroxide peroxides or superoxide radicals as the inhibitors. Finally, since BH_4 could serve as a reducing agent itself, dithrothreitol was incubated with tyrosine hydroxylase without effect, indicating that chemical reduction of the enzyme itself probably is not responsible for the loss of activity caused by the reduced cofactors.

Significance to Biomedical Research and Institute Program: These experiments indicate that the pteridine cosubstrates can stabilize tryptophan hydroxylase and the instability of this enzyme during purification may result from separation of the endogenous BH_4 from the hydroxylase. With this information, the use of thepteridines as ligands for affinity chromatography could enhance the purification of tryptophan hydroxylase because of the speed and specificity with which this process can be accomplished and it should also help stabilize the enzyme during purification. The pteridine-induced inhibition of tyrosine hydroxylase is surprising and these results indicate that, under the appropriate conditions,

some inhibitory factor is generated (suicide substrate?) which can drastically alter tyrosine hydroxylase activity.

Proposed Course of Project:

1. Continue studies on enzyme stabilization and purification using affinity chromatography.

2. Identify the substance which is inhibiting tyrosine hydroxylase.

3. Attempt to reverse the inhibitions of tyrosine hydroxylase.

Publications:

1. Kuhn, D.M., Ruskin, B. and Lovenberg, W.: Studies on the oxygen sensitivity of tryptophan hydroxylase. In: Serotonin:Current Aspects of Neurochemistry and Function, B. Hoher (ed.), Plenum Press, 1981, pp. 253-264.

2. Kuhn, S.M. and Lovenberg, W.: Structure and function of hydroxylase enzymes. In: Handbook of Neurochemistry, Vol. 4, A. Lajtha (ed.), 1981, in press.

3. Kuhn, D.M. and Lovenberg, W.: Serotonin and metabolites. In: Methods in Biogenic Amine Research, I. Najatsu and T. Nagatsu (eds.), 1981, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03510-02 HE

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Calcium-regulated Protein Phosphorylation

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

PI:	Judith Juskevich	Staff Fellow	HE NHLBI
OTHER:	Donald Kuhn	Staff Fellow	HE NHLBI
	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

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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 recently described a calcium- and calmodulin-dependent protein kinase in striatal synaptosomal cytosol. This kinase catalyzes the phosphorylation of several endogenous neuronal proteins. We are trying to further characterize this system by enzyme and substrate purification. Affinity chromatography using calmodulin-Sepharose results in a 20-fold purification of the enzyme. However, it was found that all of the major substrates for this enzyme are also retained by calmodulin-Sepharose. We are also studying the effects of endogenous proteolysis on this enzyme. Proteolysis results in a loss of sensitivity of the enzyme to calmodulin, and therefore may function in regulating the activity of this kinase.

PHS-6040
(Rev. 2-81)

Objectives: Calcium ion, through the activation of a specific calcium-dependent protein kinase, has been found to regulate the phosphorylation of specific proteins found in preparations of synaptic plasma membranes, synaptic vesicle membranes, cerebral cortical slices, synaptosomes and synaptosomal lysates. In some cases it has been shown that this calcium-dependent phosphorylation requires the heat-stable calcium binding protein, calmodulin. Since the calmodulin-regulated protein phosphorylation of synaptic vesicle proteins has been linked to the release of neurotransmitter from isolated vesicles, the protein phosphorylation system may play an important role in neuronal function.

In order to fully characterize this calcium-, calmodulin-dependent kinase, we are attempting to purify the enzyme and identify its substrates. It may then be possible to study factors affecting the activity of this enzyme and also provide information as to the physiological significance of phosphorylation of soluble proteins in nerve endings.

Methods: Male Sprague-Dawley rats were killed by decapitation. A crude synaptosomal lysate was prepared from a whole brain homogenate. The lysate was centrifuged at 140,000 x g for 60 minutes. The resulting supernatant fraction was used as a source of synaptosomal cytosol.

The net incorporation of phosphate into cytosolic proteins was determined using one of two different assays. For samples to be subjected to gel electrophoresis: cytosol was incubated at 30°C for 2 minutes in the presence of HEPES, pH 7.0, 50 mM; $MgCl_2$, 10 mM; γ^{32}P-ATP, 5 μM and in some cases $CaCl_2$, 50 μM; calmodulin, 1 μg. The reaction was terminated by solubilizing the cytosol proteins in 100 μl of a sample buffer containing 9% SDS, 0.03 M Tris HCl buffer, pH 8.0, 6% β-mercaptoethanol, 3 mM EDTA and 27% sucrose. For determination of kinase activity after column chromatography: aliquots of fractions were incubated under the same conditions as above. The reaction was terminated with 1 ml 20% cold TCA. The samples were put over millipore filters and washed twice with 10% TCA.

Proteins were resolved on SDS-polyacrylamide slab gels. The acrylamide concentration was 6% and 10% in the stacking and resolving gels, respectively. Both stacking and resolving gels contained 0.1% SDS. Following electrophoresis the gels were fixed and stained with 0.1% Coomassie blue R250 in 50% methanol, 10% acetic acid. Gels were then destained and dried under heat and vacuum. Phosphorylated proteins were located by autoradiography using Kodak RP x-ray film.

Calmodulin-Sepharose was prepared by standard methods of ligand binding to cyanogen bromide-activated Sepharose. Synaptosomal cytosol was put over a calmodulin-Sepharose column in a buffer containing HEPES, pH 7.0 20 mM; β-mercaptoethanol, NaN_3, 1 mM and NaCl 300 mM. Proteins binding to calmodulin were eluted with the same buffer with 1 mM EGTA substituted for $CaCl_2$.

Major Findings: The majority of protein in synaptosomal cytosol was found in the column effluent. Approximately 6 protein bands were visible in the eluate fraction after SDS-polyacrylamide gel electrophoresis. The calcium, calmodulin-dependent protein kinase was present in the eluate and showed an absolute dependence on the presence of both Ca^{2+} and calmodulin for activity. The activity was blocked by addition of EGTA as well as fluphenazine. All of the major substrates of this kinase were also present in the eluate fraction. This one column step resulted in a 20-fold enzyme purification.

Significance to Biomedical Research and Institute Program: Although specific phosphorylated proteins found in neuronal membranes have been implicated as physiological effectors for the diverse actions of a variety of intracellular regulatory agents, including calcium, little attention has been given to the possibility that proteins in the cytosol compartment may function in a similar capacity. That a calcium-regulated phosphorylation system in neuronal cytosol may play an important role in synaptic fuction is indicated by the observation that soluble enzymes involved in transmitter synthesis (e.g., tyrosine and tryptophan hydroxylase) are activated under phosphorylating conditions and can be regulated in a calcium-dependent manner.

Proposed Course of Project: Several other methods will be used in addition to calmodulin-Sepharose chromatography in order to purify this calcium-, calmodulin-dependent protein kinase, (for example: DEAE and affi-gel blue chromatography, molecular seive chromatography, non-denaturing gel electrophoresis). Substrates will be chromatographed and identified by two dimensional gel electrophoresis. The characteristics of this kinase, as well as the kinase-substrate interactions can then be more well-defined.

Publications:

1. O'Callaghan, J.P., Dunn, L.A., and Lovenberg, W.: Calcium regulated phosphorylation in synaptosomal cytosol: Dependence on calmodulin. Proc. Nat'l Acad. Sci. 77:5812-5816, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03511-02 HE

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

The Role of the Brain Serotonergic Neuronal System in Blood Pressure Regulation

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

PI:	Donald M. Kuhn	Staff Fellow	HE	NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE	NHLBI
	William A. Wolf	Guest Worker	HE	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.7	0.1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Microinjection of serotonin directly into the nucleus tractus solitarius produced a dose dependent increase in arterial blood pressure of anesthetized rats. The serotonin antagonists BOL and metergoline significantly attenuated the serotonin pressor effect and the serotonin uptake inhibitor fluoxetine significantly enhanced the magnitude of the pressor response. Electrical stimulation of the dorsal raphe nucleus or microinjections of serotonin into the preoptic region of the hypothalamus produced a transient rise in arterial blood pressure of both spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) controls. These effects can be attenuated by metergoline. These results demonstrate that serotonin can incluence blood pressure at a number of brain sites known to modulate blood pressure in subjects with normal or elevated blood pressure.

PHS-6040
(Rev. 2-81)

Objectives: The purpose of the present experiment was to investigate the role of the neurotransmitter serotonin in modulating arterial blood pressure by microinjecting the amine into brain sites known to exert powerful influence over blood pressure (hypothalamus, nucleus tractus solitarius) or by electrically stimulating the dorsal raphe nucleus in normotensive and genetically hypertensive rats.

Methods: Male Sprague-Dawley rats, spontaneously hypertensive, and Wistar-Kyoto rats were used. Rats were anesthetized with chloropent and one femoral artery and one femoral vein were cannulated. The subjects were then mounted in a Kopf stereotaxic instrument for stimulation. Twisted bipolar electrodes (0.25 mm diameter) were aimed at the dorsal raphe and electrical current was delivered via a WPI dual channel pulse stimulator using stimulus isolation units to ensure delivery of a constant current stimulus. The following parameters were varied at the indicated values: frequency (1, 10, 25, 50 Hz), current (100-500 μA), pulse duration (0.1-1.0 msec). Alternating biphasic squarewave pulses were delivered in trains lasting for 5 sec. Blood pressure was measured with a Statham P23ID transducer and recorded on a Sanborn Polygraph. After stimulation experiments brains were fixed in formaldehyde and electrode placement was verified histologically. Microinjections into brain structures were delivered under stereotaxic control. Injections were delivered in a volume of 0.5 μl over a period of 10-30 secs.

Major Findings: Direct microinjections of serotonin (2, 7.5, 15, 30 nmol) into the NTS of anesthetized normotensive rats produced rapid and short lived (< 15 min) increases in blood pressure ranging from 4-20 mm Hg. Heart rate did not change systematically after serotonin injections. Pretreatment of animals with the specific serotonin uptake inhibitor fluoxetine significantly increased the magnitude of the serotonin pressor effect but did not prolong it. The serotonin receptor antagonists BOL and metergoline were effective in blocking the serotonin pressor effect.

Electrical stimulation of the dorsal raphe nucleus in SHRs also produced profound effects on blood pressure. For example, increases as large as 45 and 65 mm Hg in WKY and SHR, respectively, were observed after stimulation at 500 μA. Blood pressure increased rapidly with the onset of stimulation and fell rapidly upon termination of the stimulus train. At the highest current intensities (400 and 500 μA), SHRs responded to electrical stimulation with significantly greater increases in blood pressure than WKY controls. Injections of serotonin into the preoptic area of the anterior hypothalamus (AH/PO) of SHRs produced increases in blood pressure much like that observed in normotensive rats. The pressor response to serotonin in both SHR and WKY could be blocked by the serotonin antagonist metergoline.

Significance to Biomedical Research and Institute Programs: These results extend previous studies (Kuhn et al., 1980, Hypertension 2:243-255) which established that the brain serotonergic neuronal system constitutes an important central pressor network. These data also integrate well with a growing body of evidence which has recognized the potential importance of brain serotonin in the regulation of the cardiovascular system and in the expression of certain types of hypertension. These experiments continue to provide an impetus for the investigation into the pharmacologic and brain physiologic regulation of blood pressure.

Proposed Course of Project: The following experiments will be undertaken to delineate the anatomical and neurochemical determinants of hypertension within the serotonergic neuronal system. Some of these experiments are in progress:

1. Discrete chemical lesions of the brain serotonin system will be induced with the neurotoxin 5, 7-DHT and the development of renal hypertension (DOC-salt) will be monitored.

2. The affects of increased in central nervous system serotonin on circulating renin activity will be determined.

3. The "functional" neuroanatomy of the serotonin pressor effects will be investigated by electrically stimulating the dorsal raphe nucleus while ^{3}H-2-deoxyglucose is infused.

Publications:

1. Kuhn, D.M., Wolf, W.A., and Lovenberg, W.: Pressor effects of electrical stimulation of the dorsal and median raphe nuclei in anesthetized rats. J. Pharmacol. Exp. Ther. 214:403-409, 1980.

2. Kuhn, D.M. and Lovenberg, W.: Vaso- and psychoactive substances in foods. In: Nutritional Toxicology, Nathcock, J.N. (ed.), Academic Press, 1980 (In press).

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03513-02 HE

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Effect of Cocaine on Dopamine Receptors and Dopamine-sensitive Cyclase (Revised Title)

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

PI:	Sikta Pradhan	Guest Worker	HE	NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

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 effect of cocaine on rat striatal dopamine (DA) receptors was studied in vivo and in vitro. As a part of this project, it was also our objective to determine the optimal conditions for observing the effect of cocaine on dopamine-receptor sensitivity and adenylate cyclase activity.

PHS-6040
(Rev. 2-81)

Objectives: Current literature indicates that there is a close functional relationship between dopamine receptors, adenylate cyclase, cAMP-dependent protein kinase and tyrosine hydroxylase. The objective of this study was, therefore, to test the acute effect of cocaine on dopamine-receptor sensitivity located in striatal membranes.

Methods: ^{3}H-Spiroperidol binding to rat striatal membrane was essentially carried out according to the method of Creese et al., 1978.

Adenylate cyclase was measured by a standard enzymatic method in which cAMP is generated and subsequently assayed by radioimmunoassay.

Major Findings: In prior years, we reported that cocaine administration had reciprocal effects on serotonin and substance P neurons. Cocaine also appears to influence dopamine turnover. We therefore investigated the effect of cocaine on dopamine receptor function. Five minutes following the I.P. ingestion of 20 mg/kg cocaine the apparent B_{max} of ^{3}H-Spiroperidol binding was increased by 30-40%. But the affinity of the receptor for the labelled ligands remained unchanged. Cocaine increased the apparent B_{max} of ^{3}H-Spiroperidol binding *in vitro* as well as *in vivo*. Cocaine was also observed to facilitate the responsiveness of adenylate cyclase, located in striatal membranes, to stimulation by dopamine.

Proposed Course of Project: Our previous, as well as present, results on the acute effects of cocaine in rat striatum indicate that DA-receptor sensitivity, adenylate cyclase activity and the membrane-bound form of calmodulin are involved in the kinetic regulation of TH activity. Hence to establish a mechanism of action of cocaine on the various neurotransmitter systems, it will be important to determine the changes in different neurotransmitters due to the effect of subacute and chronic treatment with cocaine.

Publications:

1) Hanbauer, I., Pradhan, S. and Yang, H.Y.T.: Role of calmodulin in dopaminergic transmission. Ann. New York Acad. Sci. 356: 292-303, 1980.

2) Pradhan, S., Hanson, G. and Lovenberg, W.: Inverse changes of SP and 5-HT in dorsal raphe following administration of serotonin-active drugs. Biochem. Pharmacol. (In press), 1981.

3) Hanson, G., Alphs, L., Pradhan, S. and Lovenberg, W.: Response of striatonigral substance P systems to a dopamine receptor agonist and antagonist. Neuropharmacology (In press), 1981.

4) Memo, M., Pradhan, S. and Hanbauer, I.: Cocaine induced supersensitivity of striatal dopamine receptors: Role of endogenous calmodulin. Neuropharmacology (In press), 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03514-02 HE

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Heat Stable Cytosolic Factors Affecting Synaptic Membrane Phosphorylation

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

PI:	Judith Juskevich	Staff Fellow	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sec. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

We have previously demonstrated the existence of a heat-stable factor in synaptosomal cytosol that regulates the phosphorylation of a particular protein in neuronal membranes. This phosphorylation is both cAMP- and calcium-independent. This factor also appears to affect phosphorylation of cytosolic proteins. Since protein phosphorylation has been linked to release of neurotransmitter, phosphorylation system may play an important role in synaptic function. It is therefore of interest to define the factors affecting the steady or dynamic phosphorylation states of neuronal proteins.

PHS-6040
(Rev. 2-81)

Objectives: We are studying factors found in synaptosomal cytosol to determine their effects on the activity of both membrane-bound and soluble protein kinases in an attempt to determine their involvement in regulation of protein phosphorylation.

Methods: A synaptosomal lysate was prepared by exposure of a crude P_2 fraction to hypotonic conditions. Synaptosomal cytosol was obtained from the lysate by centrifugation. In some experiments cytosol was put over a fluphenazine-Sepharose column to remove endogenous calmodulin.

Phosphorylation of cytosolic proteins was assayed at 30°C in a mixture containing 50 mM HEPES, pH 7.0, 10 mM or 1 mM $MgCl_2$, 1 mM DTT, 5 μM γ ^{32}P-ATP, in the absence or presence of 50 μM $CaCl_2$ and 1 μg calmodulin. After 1 minute the reaction was terminated by the addition of 100 μl of an electrophoresis sample buffer.

Proteins were then resolved on SDS-polyacrylamide slab gels. The phosphorylation of specific synaptic proteins was determined by autoradiography.

Major Findings: It was initially found in synaptic plasma membranes that calcium was required for the effects of heated cytosol on the phosphorylation of all proteins with the exception of a protein with an apparent molecular weight of 85,000 (85K band). This indicated that a heat-stable factor regulating phosphorylation, other than calmodulin, is present in synaptosomal cytosol.

We have found that the phosphorylation of cytosolic proteins can be affected by addition of phospholipids. Incubation with phosphatidylserine (5 μg) caused an increase in phosphorylation of one specific protein with an approximate molecular weight of 85K. This effect was not totally calcium-dependent. It is unclear whether this is a direct effect of phospholipids or an ionic effect, since SDS appears to have the same effect as phosphatidyl serine.

Significance to Biomedical Research and Institute Program: Phosphorylation of specific proteins found in neuronal membranes may be involved in modulating synaptic function. Phosphorylated proteins have been implicated as physiological effectors for the diverse actions of a variety of intracellular agents. Therefore, studying the regulation of protein phosphorylation may result in a greater understanding of the mechanisms involved in the regulation of synaptic function.

Proposed Course of Project: Further work will be done to characterize this calcium-calmodulin and cAMP-independent factor which stimulates protein phosphorylation. Studies will be done to determine whether this factor is a phospholipid and whether the protein affected in membranes and cytosol is the same.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03516-02 HE

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

The Biosynthesis, Distribution and Biological Role of Substance P

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

PI:	Mei-Lie Swenberg	Research Chemist	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

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)

Substance P (SP) is a neurotransmitter for neuromuscular junction and pain regulation as well as a modulator for the dopaminergic system. Studies were conducted to determine the distribution of SP in the granula of different densities from bovine adrenal medulla. The biosynthesis of SP was studied in tissue cultures of hypothalamus, substansia nigra and caudate. Studies were also conducted to investigate the effect of hypophysectomy, adrenalectomy, and splanchnic nerve denervation, on blood pressure and distribution of SP in the central nervous system (CNS) and peripheral system in normal and hypertensive rats. Specific antibodies against SP raised rabbits have been isolated and purified for the purpose of obtaining an idiotipic antibody for the purpose of identifying the SP receptors.

PHS-6040
(Rev. 2-81)

Objectives: Substance P (SP) appears to be a transmitter of major importance in mammalian brain. It has been postulated to serve a role in pain recognition, cardiovascular control (baroreflex), and as a modulator of the nigro-striatal dopaminergic system. Due to the lack of agonists and antagonists for SP, the current studies encompass several functional aspects and preparation of idiotypic antibodies:

1) Mechanisms underlying its specific biosynthesis will be investigated. These will include characterization of the precursor or intermediate precursors. Attempts will be made to complete the examination of amino acid incorporation into SP *in vivo* and in primary tissue cultures of rat brain.

2) Because of the potential role of SP in cardiovascular control, comparisons will be made between hypertensive and normal subjects in their SP levels of plasma. Studies on distribution and content will also be made on genetically hypertensive rats.

3) Effect of neurological regulation of SP biosynthesis and distribution will be investigated in genetically hypertensive rats by hypophysectomy, adrenalectomy and denervation of splanchnic nerve.

4) The potential role of the adrenal gland as a source of circulating SP will be determined by radioactive animo acid incorporation into SP *in vivo* and in tissue culture.

5) Preliminary advance study will be made for the initiation and differentiation of SP neurons and specific receptors in embryonic rats.

Methods: SP is measured by a standard radioimmunoassay with Tyr-8-SP ^{125}I labelled by the lactose peroxidase method or with SP ^{125}I labelled with Bolton Hunter's reagent. Antibodies have been generated in New Zealand white rabbits with microimmunization with SP-Bovine serum albumin conjugated with complete Freunds adjuvant at 4-6 weeks intervals. Short term tissue culture will be carried out with brain cells from various brain regions, e.g.: substantia nigra, caudate, hypothalamus, cerebellum of mature rats and the mid brain and hypothalamus of embryonic brain at different stages of gestation. Affinity column, acrylamide gel electrophoretic and high pressure liquid chromatographic methods will be applied in purification and identification of precursors, metabolites and antibodies of SP. Radioactive animo acid incorporation and organolectomy and neurolectomy will be used in search of the biosynthetic mechanism and precursors of SP *in vitro* and tissue cultures. Idiotypic antibody of SP will be prepared from purified antibody against SP raised in rabbit, by both immunization in rabbit and hybrydoma technique. Ribosomes, DNA and synthetic enzymes will be isolated from brain regions rich in SP. Release mechanism of SP from blood cells will be carried out by varying chemical factors. Blood pressure of animals was measured with tail cuff method.

Major Findings: The radioactive amino acids (proline-^{3}H and ^{35}S-methionine) reached plateau level in tissue culture of hypothalamus in 2 hours. Radioactive SP was identified in both tissue culture with higher incorporation rate for hypothalamus than substantia nigra, although, the latter tissue contain more SP.

Therefore, hypothalamus may serve as a better source for the study of SP biosynthesis and its precursors.

Distribution of SP in the various granula of bovine adrenal medulla was investigated. Similar results were obtained from two different density gradient separations of adrenal medulla cell granula with sucrose or metrizamide. Sp-like peptide was found distributed in 4 major peaks but none of them coincide with major protein peak of chromaffin granule. One exists in the leading fractions and another in the tailing fractions of chromaffin peak fractions. These results concluded that SP is present in granula of different density. Their biochemical and functional properties will be further investigated.

Acidic extraction of both blood and plasma facilitate a higher recovery of SP in human samples by the factor of 100-1000. The content of 2600 ± 200 pg/ml blood (or 24 ± 1 pg/mg protein; pg/p) and 2460 ± 1220 pg/ml plasma and 1014 ± 413 pg membrane fraction of 1 ml blood. SP-like substance was not detected in the saline wash of blood cells but a significant amount of SP-like substance was released into the saline wash containing 50 mM KCl, without the presence of Ca^{++}. After three with wash equal volume of KCl-saline the content of SP inside the cell reduced to 28 to 70% varied in different individuals. After the washing with KCl-saline, the SP content in cell membrane also changed. In some batches where the content of SP appeared to increase the membrane, the content in the wash was lower (820 ± 60 pg/ml) compared to those of 1510 ± 170 pg/ml for those with reduced SP content of the membrane. These results indicated more than one mechanism may exist in the regulation of SP release in the blood cells.

Effect of hypophysectomy, adrenalectomy and denervation of splenchnic nerve on the blood pressure and SP distribution in CNS and peripheral system was observed. In CNS the following areas were examined: Hypothalamus, cordate, substantia nigra and hypophysis; and peripheral area in adrenal and kidney. In the normotensive rats, significant increase in SP level was observed in hypothalamus. These results excluded the hypophysis as the source of SP for both CNS and peripheral system; and indicated the possibility of hypothalamus as either substitute or source of SP for hypophysis.

Substance P was found to be present in brain of embryos by the 15th day of gestation at relatively lower level; 233 ± 50, 430 ± 50, and 348 ± 120 pg/mg protein (pg/mg P) for the forebrain, cerebellum and midbrain, respectively. For comparable region on the 20th-21st day of gestation, the SP levels were 438 ± 100, 972 ± 200 and 145 ± 100 pg/mg P and for hypothalamus 1034 ± 200 pg/mg P. However, on the day after birth the level appeared to be decreased.

Specific antibodies against SP has been purified with DEAE-Affi Gel Blue to remove albumin and proteases and CM-Sephadex-C50 to remove hemoglobins. The antibodies remained active through the process. During the further purification by acrylamide, the eluant of gel corresponding to IgG band failed to show binding activity. It is possible that the antibodies were not effectively eluted from the gel or that they lost activity during the course of electrophoresis. Purified antibodies have also been subjected to HPLC with spherogel TSK column. The activity of collected fractions are currently being analyzed.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03517-02 HE

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Cardiovascular DNA and RNA Contents during Development of Cardiac Enlargement in Rats (Revised Title)

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

PI: Hisao Tanase Guest Worker HE NHLBI

OTHER: Walter Lovenberg Chief, Sect. Biochem. Pharmacol. HE NHLBI

COOPERATING UNITS (if any)

Small Animal Resources Section, Veterinary Resources Branch, Division of Research Services

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Strain and age differences in cardiovascular enlargement and nucleic acid content of the heart, were studied in SHRSP/N, SHR/N, OM/N and WKY/N rats in order to characterize cardiac enlargement. The degree of hypertrophy and hyperplasia was calculated from the difference between heart and aortic DNA concentrations. The total content between each strain and the WKY/N was almost equal to the degree of heart and aorta enlargement. SHRSP/N revealed a striking hyperplasia of myocardial cells during the pre-hypertensive stage. Hyperplasia appeared gradually with elevation in blood pressure. In contrast, the SHR/N developed a marked hyperplasia, in addition to that seen during the pre-hypertensive stage. Cardiac enlargement of the OM/N was attributed to both hypertrophy and hyperplasia. Increased heart weight was seen in the M520/N strain only at young ages, and was primarily due to hyperplasia. An increased RNA concentration was observed in both ventricles of the SHRSP/N, SHR/N and M520/N at four weeks of age, and in all four strains at sixteen weeks of age. A significantly higher RNA concentration was found in the aorta of three hypertensive strains once hypertension is established.

PHS-6040
(Rev. 2-81)

Objectives: In previous experiments, it was shown that the heart weight of rats is a highly heritable trait, and that the effect of genetic factors upon cardiac enlargement is greater than that of elevated blood pressure at a young age. In this experiment, cardiac enlargement was demonstrated in the SHRSP/N, SHR/N, OM/N and M520/N strains. Cardiac enlargement in these strains might be due to different factors since their gene constitutions are apparently different. Therefore, the present experiments deals with strain and age differences in the amount of cardiac and aortic DNA and RNA in an attempt to characterize cardiac enlargement in these strains as compared with the WKY/N.

Methods: The SHRSP/N, SHR/N, OM/N, M520/N and WKY/N strains were obtained from the NIH small animal resources. At 4, 8, 12 and 16 weeks of age, their body weight, blood pressure and heart rate were measured. After that, the heart and aorta were removed and weighed. Then, DNA and RNA in both ventricles and aorta were extracted by PCA and assayed according to the methods of Ceriotti and Dische, respectively.

Major Findings: The experiments indicated that the sum of the degree of hypertrophy and hyperplasia (calculated from cardiac or aortic DNA concentration) and total content is almost equal to the degree of cardiac or aortic enlargement. Therefore, from the viewpoint of cellular hypertrophy and hyperplasia, it will be possible to characterize the enlargement of heart and aorta in each strain as compared with the WKY/N. The SHRSP/N revealed a striking hypertrophy of myocardial cells from the pre-hypertensive stage, and hyperplasia appeared gradually with the elevation of blood pressure. In contrast, the SHR/N developed a marked hyperplasia and in part hypertrophy from a pre-hypertensive stage. Cardiac enlargement of the OM/N was attributed to both hypertrophy and hyperplasia. A large heart weight of the M520/N was recognized at only young age, and was due mainly to hyperplasia. Aortic enlargement was almost totally related to hyperplasia. An increased RNA concentration was observed in both ventricles of the SHRSP/N, SHR/N and M520/N at four weeks of age, and all of four strains at sixteen weeks of age. A significantly higher RNA concentration was indicated in aorta of three hypertensive SHRSP/N, SHR/N and OM/N strains at established hypertensive stage. These changes might be related to the manifestation of genetic or other factors as well as effect of elevated blood pressure.

Significance to Biomedical Research and Institute Programs: Cardiac hypertrophy caused by overload may be due to an increase of cell size (hypertrophy), to an increment in cell number (hyperplasia), or to both. The current study provides the possibility that cardiac enlargement in human beings could be distinguished as to hypertrophy and hyperplasia to a certain extent by measuring DNA content.

Proposed Course of Project: Two aspects of previous and present studies will be pursued. (1) RNA determinations reflect changes in synthesis of cellular components such as myosin and collagen. Therefore, further comparative biochemical studies in these strains will be possible in order to characterize cardiac enlargement. (2) An effort will be made to develop hypertensive and normotensive strains accompanied with large or small heart by the selection experiment from the hybrids among these strains as a base population. These new four strains could be contributed to comparative study of cardiac enlargement as more useful animal models.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03518-01 HE

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Serotonin Receptors and Cardiac Ventricular Sarcolemma

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

PI: Elliott Kulakowski Staff Fellow HE NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.3	PROFESSIONAL: 0.3	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 high affinity serotonin receptor has been identified on the cardiac ventricular sarcolemma. Serotonin analogues compete for this binding site but classical serotonin antagonists are weak inhibitors. At high concentrations, catecholamines are able to displace serotonin binding but this inhibition does not appear to be related to serotonin binding to adrenergic receptors.

PHS-6040
(Rev. 2-81)

Objectives: Serotonin (5-HT) exerts a direct, positive inotropic effect on the rat heart. In order to determine the mechanism by which serotonin brings about this response, we investigated the interaction of serotonin with the cardiac sarcolemma. The studies consisted of: 1) examine for the presence of a discrete serotonin receptor, 2) studying the effects of serotonin on sarcolemma enzymes, and 3) determining if serotonin plays a role in ion movement across the sarcolemma.

Methods: Sarcolemma Preparations. Male Sprague Dawley rats (200-250 gms) were used in all experiments. They were housed at the N.I.H. animal facilities and given food and water ad libitum. The hearts were removed from the rats and perfused retrogradely, within 60 seconds, with 0.25 M ice cold sucrose to remove blood from the ventricles and coronary vessels. The ventricular tissue was isolated, weighed and minced in 5 volumes (w/v) 0.25 M ice cold sucrose. The tissue was homogenized at $4^{o}C$ in a Waring Blender and centrifuged at 600 x g for 10 minutes at $4^{o}C$. The supernatant and loose pellet layer were collected and centrifuged at 2000 x g for 10 min. The pellet was washed three times by suspension in 10 mM Tris-HCl, pH 8.0 and centrifuged as described above. The final pellet was suspended in buffer and 4.0M LiBr was added slowly, with stirring, to yield a final concentration of 0.4M LiBr. The suspension was stirred for 1 hr at $4^{o}C$, diluted four fold with buffer and passed through eight layers of cheese cloth. The filtrate was centrifuged at 2000 x g for 10 minutes at $4^{o}C$ and the pellet washed with 10 mM Tris HCl, pH 8.0. The LiBr extracted particles were suspended in 10 mM Tris-HCl, pH 7.5 containing 25% KBr and centrifuged for 30 minutes at 7000 x g. The pellet was washed three times with 10 mM Tris HCl, pH 8.0 and the final pellet was suspended in 10 mM Tris HCl, pH 7.4 containing 120 mM NaCl, 1.25 mM $CaCl_2$, 4.8 mM KCl, 1.2 mM $MgSO_4$ and 1.2 mM KH_2PO_4.

Binding Assay. The binding of 5-hydroxytryptamine to the isolated cardiac ventricular sarcolemma was determined using freshly prepared membranes. Membrane (80-100 μg sarcolemma protein) was preincubated at $24^{o}C$ for 15 minutes in the ion-containing buffer prior to the addition of $[^{3}H]$-serotonin (32.5 Ci/mmol). The reaction was terminated after one-hour incubation by the addition of 3 ml ice cold buffer. The membranes were isolated by filtration on Whatman GF/B glass fiber filters and washed three times with 3 ml volumes of ice cold buffer. The filters were placed in scintillation fluid and counted in a Beckman LS-8100 liquid scintillation counter. Specific binding was determined as the difference between total binding and non-specific binding, which was determined in the presence of at least 1000 fold excess of serotonin.

Major Findings: A discrete serotonin receptor is present on the rat cardiac ventricular sarcolemma. Binding of serotonin over the concentration range 1-150 nM was non-cooperative as determined from the Hill coefficient of 0.93. Scatchard analysis of the binding data reveals a dissociation constant (K_D)=48.8 nM and a Bmax=12.2 p moles/mg sarcolemma protein. This data compares favorably with a K_D= 45.6 nM determined the rate contants for association and dissociation.

Serotonin binding to cardiac ventricular sarcolemma is inhibited by serotonin analogues but not by the classical anti-serotonin agents. The order of potency for the serotonin analogues is 5-hydroxyindoleacetic acid > 7-dihydroxytryptamine > 5-hydroxytryptamine 5-methoxytryptamine > tryptophan > tryptamine. Based on this order of potency the 5-hydroxy moiety appears to be necessary for binding. In addition to LSD and its analogues, cyproheptadiene, methiothepin and

cinanserin fail to appreciably alter serotonin binding while mianserin only inhibits binding at 40% at 2000 fold excess.

Catecholamine, at a concentration greater than 10^{-7}M, exhibits cross reactivity for the cardiac 5-HT binding site. The adrenergic agents isoproterenol, epinephrine, nonepinephrine and phenylephrine have IC_{50} values of 7, 40, 60 and 100 μM respectively when compared to 48 mM serotonin. However, this inhibition by catecholamines appears to be unrelated to either α- or β-adrenegic receptors since neither propranolol nor phentolamine inhibit serotonin binding. Furthermore, if serotonin acted through the β-adrenergic receptor, then 5-HT should stimulate adenylate cyclase. However, no activation of adenylate cyclase was observed.

The serotonin receptors on rat heart sarcolemma are observed over the same concentration range that is necessary to elicit a positive inotropic effect. The action of 5-HT is not mediated through α or β adrenergic receptors and is not related to cAMP production. However, serotonin may function through activation of the sarcolemma ATPases or alter calcium binding or flux in the heart. These parameters are currently being investigated.

Significance: Serotonin, is synthesized and stored in high concentrations in the heart similar to the catecholamines. Thus, since both serototin and catecholamines produce a positive inotropic response they may both play an important role in the stressed myocardium. Our results indicate that; 1) serotonin binds to discrete serotonin receptors, 2) these binding sites are not related to adrenergic receptors, 3) since it does not activate adenylate cyclase its action may be on other membrane proteins or related to ion movements.

Little is known about the interaction of 5-HT with the sarcolemma. Even less is known about the mechanism by which serotonin induces a positive inotropic response. A better understanding of the mode of action of 5-HT would help to assign physiological and pathological roles for 5-HT and perhaps suggest new treatments for the stressed myocardium.

Proposed Course: An examination of the effects of serotonin on sarcolemma proteins and calcium movements will be investigated. Various concentrations of serotonin will be added to membrane preparations to see if there is a change in Na^+K^+-ATPase, Mg^{++}-ATPase, or Ca^{++} ATPase. In addition, calcium binding and uptake experiments are proposed to determine if serotonin has any effect on calcium movements across the sarcolemma. Any stimulatory effect of calcium will be characterized in the presence and absence of calcium antagonisits such as verapamil and lanthanum chloride.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03519-01 HE

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Analysis of 6-Phenylpterin as a Cofactor for Various Hydroxylases from Mammalian Tissue

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

PI:	Leonard Miller	Guest Worker	HE NHLBI
OTHER:	Anne Culvenor	Guest Worker	Fogarty International Center

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.25	0.25	

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

-Tetrahydropterin forms the basic structure of series of analogues which serve effectively as cofactors for hydroxylase enzymes. Most analogues are formed by placement of various side chains on the 6-carbon. We recently acquired a compound which has a phenyl group attached at this 6-position. The present investigation compared this compound with tetrahydrobioptin for effective hydroxylation of phenylamine, tyrosine and tryptophan. The enzymes were phenylalanine hydroxylase purified from rat liver and tyrosine and tryptophan hydroxylase from rat brain. In addition we examined the ability of the reductase system to continually regenerate reduced 6-methylpterin. Our kinetic analysis showed that 6- methylpterin serves as effective as the natural cofactor (tetrahydrobiopterin) for the various hydroxylases. Also, 6-methyl-pterin is incorporated effectively into the endogenous reductase system.

PHS-6040
(Rev. 2-81)

Objectives: To determine if the analogue 6-phenyltetrahydropterin can function effectively as a cofactor for the hydroxylase enzymes.

Methods: Tyrosine, phenylalanine and tryptophan hydroxylase were determined by present ongoing procedures within the laboratory. 6-phenyltetrahydropterin was supplied by Dr. Carl Storm at Howard University.

Major Findings: 6-phenylpterin is as effective a cofactor as tetrahydropterin for all the three hydroxylases examined. In addition, 6-phenylpterin is effectively incorporated into the reductase system which is responsible for keeping the cofactor in the reduced form.

Significance to Biomedical Research: The present investigation represents the first step in determining the usefulness of this substance for incorporation into hydroxylase processes.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03520-01 HE

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Dopamine Receptor Regulation in Schizophrenic Illness

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

PI:	Maurizio Memo	Guest Worker	HE NHLBI
OTHER:	Ingeborg Hanbauer	Pharmacologist	HE NHLBI
	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Studies on adenylate cyclase in striata and nucleus accumbens of control and schizophrenic brains were carried out. The adenylate cyclase activity of both brain areas in schizophrenics was more enhanced by NaF than in controls. In contrast, dopamine stimulated adenylate cyclase in controls and schizophrenics to the same extent.

PHS-6040
(Rev. 2-81)

Objectives: The "dopamine hypothesis" of schizophrenia evolved from the finding that dopamine releasing agents such as amphetamine induce schizophrenic-like symptoms whereas dopamine receptor antagonists, such as butyrophenones or phenothiazines alleviate these symptoms. In support of this proposal, it has been shown that there is an increased density of striatal dopamine receptors in schizophrenic post-mortem brain samples.

There is now evidence that two classes of dopamine receptors are present in the CNS : D_1, associated, and D_2 not associated with adenylate cyclase activity. Recent studies propose that dopamine receptors function as a supramolecular entity and that receptor activity results from an orderly molecular interaction of a number of membrane proteins. In the case of D_1 receptors, the flow of informations across the membrane proceeds from the recognition sites by the way of a coupling system to the enzyme adenylate cyclase. The coupling system of D_1 receptors includes GTP-binding protein and calmodulin.

Methods: The molecular properties of D_1 dopamine receptors were studied in striatum and nucleus accumbens from autopsied brains of 3 schizophrenic and 8 non-schizophrenic individuals. In particular we measured the stimulation by DA or NaF of adenylate cyclase activity in striatum and nucleus accumbens. Since neuroleptic drugs which may be still present in schizophrenic brain could interfere with DA stimulation of adenylate cyclase, we also measured the stimulation of adenylate cyclase by NaF. Adenylate cyclase activity was carried out by the method described by Clement-Courmier (Proc. Natl. Acad. Sci., USA, 71: 1113-1117, 1974).

Major Findings: The maximal stimulation of adenylate cyclase activity elicited by 10 mM NaF is increased by about 50% in striatum from schizophrenics in comparison to that from controls. In contrast, the ED_{50} of NaF for adenylate cyclase is similar in striatum of schizophrenics and controls. Kinetic analysis indicate an increase in V_{max} of the enzyme. When DA was used as stimulant, neither the V_{max} nor the K_D of striatal adenylate cyclase from schizophrenics or controls was altered. Preliminary data indicate that also in nucleus accumbens from schizophrenics, NaF increased the V_{max} more than in controls, suggesting that in different dopaminergic brain areas of schizophrenics, adenylate cyclase may be supersensitive to NaF. The finding that DA acts in the same order of magnitude in striatum of schizophrenics and controls might be due to residual neuroleptic drugs.

Proposed Course of Action: Future experiments are planned to better characterize the D_1 dopamine receptor alterations in schizophrenia. In particular, studies on calmodulin compartmentation and response of adenylate cyclase to cholera toxin are in progress.

In view of the existence of multiple classes of dopamine receptors it would be of interest to see if other dopamine receptors are involved in schizophrenic illness. All these studies will be conducted in striatum and nucleus accumbens from autopsied brains of schizophrenic and non-schizophrenic individuals.

Significance of Biomedical Research.

This research project is designed to obtain a better understanding of biochemical defects that may exist in schizophrenic illness. Better insight on the abnormalities of dopaminergic transmission will allow more rational, specific and effective therapy.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03521-01 HE

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Regulation and Turnover of Tetrahydrobiopterin

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

PI:	Anne Culvenor	Guest Worker	Fogarty International Center	HE NHLBI
	Leonard Miller	Guest Worker		HE NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

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NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.5	PROFESSIONAL: 1.5	OTHER:

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The rat cultured pineal gland was set up in order to study tetrahydrobiopterin (BH_4) regulation and turnover. When pineals from 250 g rats were cultured, pineal BH_4 decreased to 50% of control levels after 24 hours of culture. This was due to release of BH_4, detected as biopterin, into the culture medium. A suitable culture system was obtained when better tissue oxygenation was achieved by using smaller pineals from 100 g rats. Under these conditions pineal BH_4 remained stable for 24 hours in culture, with an increase at 48 hours to 150% of initial levels, and less BH_4 was released into the culture medium. Pineal levels of the cellular marker, lactate dehydrogenase (LDH) were constant during culture, indicating that most of the pineal cells remained viable. The ratio of reduced to oxidized biopterin did not change during culture, suggesting that BH_4 regeneration from quinonoid dihydrobiopterin (q-BH_2) was occurring normally in the cultured glands. In contrast, cultured murine P815 mastocytoma cells were not a suitable system since BH_4 levels fluctuated and cells appeared to multiply during culture. A biopterin-bovine serum albumin conjugate is being used as an immunogen in rabbits to develop antibodies to biopterin, ultimately required for the development of an immunoassay for biopterin.

PHS-6040
(Rev. 2-81)

Objectives: The main aim of these experiments was to develop organ and/or cell culture systems as models to study the regulation and turnover of BH_4 and its role in monoamine synthesis. A minor aim was to develop an immunoassay for biopterin.

Methods: Pineals were obtained from male Sprague Dawley rats and cultured for up to 48 hours. Murine mastocytoma P815 cells were collected from the peritoneal fluid of male DBA-2 mice, washed, and cultured for up to 32 hours. In early experiments BH_4 was measured by the coupled radioenzymic method of Levine et al. (J. Neurochem., 32: 1575-1578, 1979). In later experiments oxidized and reduced forms of bipterin were assayed by differential oxidation and high performance liquid chromatography (HPLC) method of Fukushima and Nixon (Anal. Biochem., 102: 176-188, 1980). LDH was measured by the method of Kornberg (Methods Enzymol., 1: 441-443, 1965).

Major Findings: Using the coupled radioenzymic assay, BH_4 was measured in pineals obtained from 250 g rats and cultured for up to 48 hours. Following 24 hours of culture, pineal BH_4 decreased to 50% of initial levels and remained stable for a further 24 hours. The initial decline was not due to degeneration of noradrenergic terminals originating from the superior cervical ganglion, since superior cervical ganglionectomy does not significantly alter rat pineal BH_4 levels.

When the HPLC assay for biopterin was adapted to measure total biopterin in the culture medium, the 50% decline in pineal BH_4 during the first 24 hours of culture was reflected by the appearance of an equivalent amount of biopterin in the culture medium, presumably due to release of BH_4 from the pineal and subsequent oxidation in the medium.

When modifications were made to the culturing procedure to ensure better tissue oxygenation, including the use of smaller glands from 100 g rats and a reduction in culture volume, pineal BH_4 remained stable for 24 hours in culture, with an increase at 48 hours to 150% of initial levels. Less BH_4 was released into the medium under these conditions. The ratio of reduced to oxidized biopterin did not change significantly from control (about 4:1) throughout the culture period. This suggests that the quinonoid dihydropteridine reductase system which regenerates BH_4 from q-BH_2 was functioning adequately during culture. Measurement of the cellular marker enzyme LDH as an estimate of pineal cell viability showed that there was no significant change from control in pineal LDH during culture. Therefore, the modified rat pineal culture system seemed to be suitable for the study of BH_4 regulation and turnover.

When washed mastocytoma cells were cultured for up to 32 hours in BGJ medium, there was an initial decline of about 25% relative to control in cell BH_4 at 2 and 4 hours of culture, followed by a return to control level at 6 hours. BH_4 levels then increased at 10 hours and remained elevated for up to 32 hours (200% of control at 25, 32 hours). Cell protein and cell volume density were also significantly increased at the later times, suggesting that cell multiplication or hypertrophy was occurring during culture.

Culture of the mastocytoma cells in a less supportive growth medium was then attempted, but BH_4 levels dropped to zero by 24 hours, probably indicating cell death. Since it appeared to be difficult to obtain a stable short-term culture of mastocytoma cells, it was felt that this was not a suitable system for the study of BH_4 regulation and turnover.

Biopterin was coupled to bovine serum albumin as an immunogen to be used in the production of antibodies for the immunoassay of biopterin. The low solubility of biopterin at the pH required for coupling to proceed resulted in a low degree of conjugation of biopterin to protein.

Although rabbits are being immunized with the conjugate, its low epitope density makes it probable that many immunizations over a long period will be necessary to obtain antibodies.

Significance to Biomedical Research and Institute Programs: This project aims to set up organ and cell cultures as model systems for monoaminergic neurons. The use of a relatively simple system such as the rat pineal gland should aid in a further understanding of the mechanisms which regulate BH_4 levels and turnover in monoaminergic neurons. In addition these studies should help to clarify the potentially important role of BH_4 in the regulation of monoamine synthesis.

Proposed Course: The principal investigator has applied for a 12 month renewal of her fellowship, currently due to finish at the end of August, 1981. Therefore, it is anticipated that the project will continue untl the end of August, 1982.

In future experiments, the turnover of BH_4 in the cultured pineal gland will be measured by incorporation of radioactive precursors into BH_4, using HPLC to separate labelled compounds and quantitate BH_4. The activity of the enzyme, GTP cyclohydrolase, which catalyzes the apparent rate-limiting step in the synthesis of BH_4 from GTP, will also be measured in the cultured glands. This will enable a simultaneous investigation of the effect of drugs and precursors on the steady state level and turnover of BH_4 and on the rate of monoamine synthesis in the same system.

As an alternative model system, the PC-12 cell line, cloned from transplantable rat phaeochomocytoma, will be cultured for BH_4 regulation and turnover studies. This should prove a valuable system to study the effects of nerve growth factor (NGF) on BH_4, since PC-12 cells respond to NGF by differentiation and acquire many of the properties of sympathetic neurons.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03522-01 HE

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Penetration of Biopterin and Other Analogues into Rat Brain Tissue

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

PI:	Leonard Miller	Guest Worker	HE NHLBI
OTHER:	Glenn Robinson	Summer Student	HE NHLBI
	Elliott Kulakowski	Staff Fellow	HE NHLBI
	Robert Levine	Pharmacologist	HE NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	0.75	0.25

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The present investigation analyzed the ability of tetrahydrobiopterin (BH_4) and 6-methyltetrahydropterin (6 mPH_4) to penetrate into rat brain tissue following peripheral administration. The drugs were given i.p. at a dose of 100 mg/kg. Following injection of BH_4 or 6 MPH_4, 5 different areas of rat brain were removed at 5 different time periods to be analyzed for levels of BH_4 and 6 mPH_4 respectively: striatum, hippocampus, hypothalamus, cortex and cerebellum: time points - 0, 30, 60, 90, 120 and 150 min. post injection. At present we see no change in the endogenous levels of striatal BH_4 following i.p. BH_4. However, there is a significant increase in 6 MPH_4 following its i.p. injection. We will subsequently examine the effect of 6 mPH_4 injection on endogenous dopamine synthesis by monitoring levels of the metabolites DOPAC and HVA. If these results prove effective we will then examine for increased levels of dopamine neurotransmission.

PHS-6040
(Rev. 2-81)

Objectives: To determine if tetrahydrobiopterin or one of its analogues will penetrate the blood-brain barrier following peripheral administration and result in increased dopamine synthesis.

Methods: Tetrahydrobiopterin (THD) and 6-methylpterin (MPH_4) will be administered i.p. and at the indicated time period rat killed, brain regions removed and analyzed for THB and 6-MPH_4 by HPLC-fluorescence. DOPAC, HUA and Dopamine will be analyzed by HPLC-electrochemical detection. Turning of nigral-striatal lesioned animals will be analyzed on a rotometer.

Major Findings: THB administered i.p. at a dose of 100 mg/kg has no significant effect on striatal THB levels. 6 MPH_4 administered at a dose of 100 mg/kg shows a significant rise in the rat striatum within 30 min and up to 150 min of i.p. injection. Most of the compound in striatum appears present as the tetrahydroform. Striatal samples will soon be analyzed for DOPAC, HUA and Dopamine levels.

Significance to Biomedical Research: This project seeks to determine the efficacy of THB or one of its analogues on striatal dopamine neurotransmission following peripheral administration.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03523-01 HE

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Administration of Tetrahydrobiopterin to Parkinson's Patients

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

PI:	Leonard Miller	Guest Worker	HE NHLBI
OTHER:	Peter Lewitt	Clin. Assoc.	ET NINCDS
	Donald Calne	Clin. Dir.	IR NINCDS
	Walter Lovenberg	Chief, Sect. on Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

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)

Degeneration of the nigral-striatal dopaminergic pathway is a major characteristic of patients with Parkinson's disease. Presently the etiology of this progressive degenerative process is unknown. However, the symptoms of this disorder can apparently be remedied by increasing neurotransmitter levels in the surviving neurons. This is accomplished by administering high levels of the precursor L-Dopa. The present investigation is an attempt also to increase endogenous dopamine neurotransmission by increasing the metabolism of the rate-limiting enzyme tyrosine hydroxylase. Apparently this can be accomplished by raising the in vivo tissue levels of tetrahydrobiopterin (BH_4). Our approach will be to administer large doses BH_4 (2.5 mg/kg) of BH_4 to patients while monitoring physiological responses as indicated in the protocol. Concurrent with this, CSF biopterin levels, DOPAC and HVA will be determined.

PHS-6040
(Rev. 2-81)

Objectives: The major objective of this project is to determine whether patients with neurological disorders related to CNS dysfunction can be successfully treated with the endogenous cofactor tetrahydrobiopterin or an analogue thereof.

Methods: The project involved focusing on two different but overlapping approaches:

A) Administration of tetrahydrobiopterin or one of its analogues to monkey following by analysis of CSF amine metabolites in addition to tetrahydrobiopterin levels.

B) Administration of tetrahydrobiopterin to Parkinsonian patients with a complete neurological workup to determine the effectiveness of this approach.

Major Findings: At present the drug tetrahydrobiopterin has passed through the screening process of the NIH Pharmacology Development Section. Monkey CSF shows higher levels of biopterin than human. Procedural approval has come from the FDA such that administration of the drug should proceed by the end of July '81.

Significance to Biomedical Research and Institute Programs: This project seeks a new approach to the treatment of Parkinson's disease by making use of substances endogenous to the body.

ANNUAL REPORT OF THE
LABORATORY OF KIDNEY AND ELECTROLYTE METABOLISM
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
OCTOBER 1, 1980 through SEPTEMBER 30, 1981

Our continuing goal is to analyze the function of the kidney as a basis for understanding its pathophysiology and treating its disorders. Since the formation of urine depends upon the transport of water and solutes by kidney tubules, understanding renal function requires analysis of these cellular processes and of their integration in the kidney. Therefore, we are studying transport by cells in general and kidney cells in particular, as well as the mechanisms, hormonal and other, that control transport.

Isolated segments of renal tubules.

In order to understand kidneys on the cellular and molecular level the functions of the different types of epithelial cells must be identified. Progress in this direction has relied heavily on the direct study of individual nephron segments. Each nephron segment has a different cell morphology and function. An important method (which originated in this laboratory) for directly studying the nephron segments is to dissect them and perfuse them individually *in vitro*. The findings, during the past year, using this method are as follows:

1. Urea transport in isolated segments of rabbit renal tubules.

Effective concentration of the urine requires that urea accumulate in the renal medulla to concentrations far above that of plasma. The mechanisms involved in urea accumulation are not completely understood. A pathway for urea recycling between collecting ducts and ascending limbs has been described. Another pathway for urea recycling may exist, however, between the ascending and descending limbs. For this to be possible, urea must be absorbed from the ascending limb of Henle at some level. Knepper and Vurek are studying urea transport across thick ascending limbs. They have found significant passive permeability to urea, but no evidence for active urea transport. The investigators propose that urea may diffuse out of the thick ascending limbs in vivo into adjacent proximal straight tubules. They next intend to test whether proximal straight tubules take up urea, as is necessary in this theory.

2. Segmental localization of renal kallikrein.

The renal kallikrein-kinin system may play a role in the regulation of NaCl excretion. As the first step in the investigation of the effects of kallikrein and kinins on nephron sodium chloride transport, studies have been initiated by Knepper in collaboration with Pisano and Proud (Laboratory of Physiological Chemistry) to determine which nephron segments possess kallikrein activity measurable by radioimmunoassay. Initial experiments were carried out with homogenized tissue slices from different levels in the rat kidney.

The highest kallikrein content was found in the outer cortex. The kallikrein content was lower in the inner cortex. Small but definite amounts of kallikrein were also found throughout the medulla. Based on this information they will now attempt to assay kallikrein in individual tubule segments dissected from the regions with the highest concentration and thus localize the system within the nephron.

3. Acidification and bicarbonate transport by renal tubules.

Atkins and Burg are studying acidification by proximal straight tubules. Previously McKinney and Burg measured bicarbonate absorption in this segment and found it was rapid, but that the tubule fluid bicarbonate concentration did not fall on the average below a steady-state value of 10 mM, corresponding to a transepithelial pH difference of only 0.5 units. By contrast, the pH difference between blood and urine in rabbits may exceed 2 units and bicarbonate may be totally absent from the urine. The purpose of the present studies was to test different conditions that might affect the H^+ concentration difference across proximal straight tubules. pH was measured in the perfusate, collected tubule fluid and bath. With normal levels of bicarbonate and CO_2 in the bath, the steady state transepithelial pH difference was 0.5 units, consistent with the earlier measurements of bicarbonate concentration. When the bath was free of bicarbonate and carbon dioxide, the result was the same. Other combinations of bath pH, bicarbonate, and carbon dioxide concentrations are being tested to determine the importance of each of these factors.

Good and Burg are studying bicarbonate transport by isolated, perfused rabbit cortical collecting ducts. Previously, McKinney and Burg had found that collecting ducts from alkalotic rabbits (but not acidotic ones) secreted bicarbonate. The purpose of the present studies is to identify factors affecting the bicarbonate secretion. 8-bromo cyclic AMP, isobutyl-methyl-xanthine, isoproterenol, and sodium-free conditions (cesium replacement) are being tested.

4. Function of connecting tubules.

The connecting tubule is the part of the distal nephron between the distal convoluted tubule and cortical collecting duct. It is distinguished from the cortical collecting duct by subtle microscopic differences. Almeida and Burg are studying electrical resistance and sodium transport by isolated, perfused rabbit connecting tubules. They found that the voltage was approximately -20 mV, lumen negative. The transepithelial electrical resistance was relatively low (35 ohm cm^2 vs 250 ohm cm^2 in cortical collecting ducts). Transepithelial fluxes of ^{22}Na both into and out of the lumen were high, as was the net sodium flux which was 3-4 times greater than that in the cortical collecting duct. Isoproterenol decreased the voltage, but did not affect sodium flux. Studies are now under way to test whether changes in transport of other ions, such as chloride, bicarbonate, or potassium might account for the voltage change following isoproterenol.

Cell culture of kidney and other urinary epithelia.

Although the technique of perfusing kidney tubules *in vitro* has provided an overall description of their transport properties, it has been difficult to extend the studies to subcellular and molecular levels. Chemical and physical methods for studying transport require much larger amounts of homogeneous tissue than are present in single tubules. Dr. Handler and his colleagues have been using culture of epithelial cells to overcome this difficulty.

Moran and Turner prepared apical membrane vesicles from $LLC\text{-}PK_1$, a cell line derived from pig kidney. The cells form an epithelium that transports glucose in a sodium-dependent manner, resembling the proximal tubule. The investigators identified sodium-coupled glucose transport in the vesicles. They will next measure the coupling ratio between sodium and glucose, and other characteristics of the system.

Burg, Sohraby, Green and their associates have continued to use collagen membranes prepared by Steele's technique (see last year's report) to culture cells from dissected segments of the medullary thick ascending limb. Primary cultures form confluent epithelia with a transepithelial voltage oriented apical surface positive. The potential difference is reduced by the addition of furosemide to the solution bathing the apical surface, whereas amiloride has no effect. The orientation of the voltage and the effects of the drugs are like those observed with medullary thick ascending limbs. Attempts to propagate lines of cells that continue to manifest these differentiated properties have not been successful so far but are continuing as are attempts to culture epithelia from other nephron segments.

The cell lines developed by Handler et al. from toad urinary bladder are being used to study the interaction of thyroid hormone and aldosterone. In one cell line, TB6C, thyroid hormone prevents the sodium transport response to aldosterone. In another line, TB-M, it does not. In order to test the site of thyroid hormone action methods have been developed to assay cytosolic aldosterone receptors and nuclear receptor-bound aldosterone. The effect of thyroid hormone on aldosterone receptors will be examined next.

A6, a line of cells derived from toad kidney, forms an epithelium with many functions similar to collecting duct. Sodium transport by A6 is known to be stimulated by at least three hormones aldosterone, vasopressin and insulin. Preston and Handler have now found that epinephine also stimulates sodium transport.

A6 cells grown on porous supports were found to be more differentiated than when grown on plastic dishes. The differentiation included increased ciliary action and induction of a sodium transport response to vasopressin. The investigators attributed the effects to better access of the nutrient solution to the base of the cells through the porous support, analogous to the normal nutrition in vivo. Steele and his associates are now developing better techniques

for growing epithelia on porous supports in order to improve basal nutrition, cause further differentiation, and measure transepithelial transport more easily. One improvement has been to grow the cells on millipore filters which are more permeable than the nucleopore filters used in the past. Another is to eliminate serum from the medium bathing the apical surface of the epithelium while retaining it on the serosal surfaces, as in vivo. This was found to inhibit cell overgrowth.

Transport of D-glucose by proximal tubule brush border membranes.

D-glucose is reabsorbed from the urine by the renal proximal tubule. The active step is D-glucose co-transport with sodium into the cells across the lumen brush border membrane. Turner is studying this process in membrane vesicles since, in addition to its obvious physiological significance, the brush border membrane D-glucose transporter is a useful model system for studying the mechanism of co-transport processes in general. Turner and Moran are comparing glucose transport in brush border membrane vesicles prepared from the outer cortex (early proximal tubule) and outer medulla (late proximal tubule) of rabbit kidney. They have established that the glucose transport properties of these two preparations are markedly different. Preparations from the outer cortex exhibited a low affinity (K_m 3.4 mM), high capacity transport system while preparations from the outer medulla showed a high affinity (K_m 0.6 mM), low capacity system. In the outer cortical transporter the stoichiometry was 1:1:1 (phlorizin:glucose:sodium) i.e., one phlorizin molecule competes with one glucose molecule for a site on the transporter and this "substrate site" is associated with a single sodium binding site. Preliminary results of similar experiments on the outer medullary preparation indicate a sodium:glucose stoichiometry of greater than one. The differences correlate well with the known distribution and characteristics of glucose transport in the tubule. The earliest part of the proximal tubule (predominant in the outer cortex) transports glucose rapidly but does not establish large concentration differences while the later part in the outer medulla transports less glucose but establishes large concentration differences, virtually eliminating glucose from the urine. Further experiments are planned to elucidate the molecular basis for the kinetic differences between the transporters.

Isolation and study of brush border membrane transport proteins.

Recently, several primary active transport proteins have been extracted from cell membranes and purified to near homogeneity by the application of conventional protein separation techniques. Efforts to isolate transporters that couple ion and/or nonelectrolyte fluxes have been less successful. One reason is that, unlike primary active systems, coupled transporters do not typically exhibit an easily measured enzymatic activity to follow through the various extraction and separation steps. Turner is attempting to purify the sodium-coupled glucose transporter using monoclonal antibodies. His first objective is to produce a series of specific antibodies against the brush border membrane transporter and to use these antibodies to

purify selectively the transporter from a detergent extract of the membrane. Turner has immunized mice with rabbit renal brush border membranes and has carried out a successful spleen cell to myeloma cell fusion. Five hybrid cell lines have been selected for future study on the basis of the apparent ability of the antibodies they produce to inhibit or stimulate phlorizin binding to brush border membrane vesicles. In parallel with the monoclonal antibody work Turner is attempting to obtain purified preparations of the brush border membrane D-glucose transporter using conventional detergent extraction and separation techniques. These preparations can be used in turn as antigens for preparing monoclonal antibodies and as a more selective means of detecting them.

Non-invasive studies of renal cellular energetics.

Active ion transport constitutes up to 70% of the work performed by the mammalian kidney. Balaban and Orloff are studying the coupling of metabolic energy conversion to active ion transport in the kidney using the non-invasive tools of nuclear magnetic resonance (NMR) and optical spectroscopy. These techniques permit the direct monitoring of metabolic intermediates (i.e., ATP, ADP, mitochondrial cytochromes etc.) within the cell during active ion transport. The techniques have already been applied to isolated perfused tubules, renal tubule suspensions, and the kidney *in vivo*. Procedures for studying renal cell culture lines by NMR are being developed in collaboration with Dr. Richard Knazek, CIP. The general aim of all these studies is to learn the control sites which balance the rate of energy conversion with the rate of energy utilization by active ion transport. Transformed cells are also being studied in collaboration with Dr. John Bader, NCI. In those cells the control sites and coupling appear to be markedly different from normal cells which may be an important aspect of transformation.

Necturus gallbladder epithelium.

The cellular aspects of epithelial transport can be studied directly by making measurements in individual cells and in the spaces between them. Since mammalian cells generally are very small, such direct studies are difficult and there are artifacts due to cell damage. Therefore, Spring and his coworkers have been using the relatively large gallbladder cell of *Necturus* for this purpose.

(1) NaCl entry into gallbladder epithelial cells.

Ericson and Spring demonstrated that inhibition of active sodium transport out of the base of *Necturus* gallbladder cells by ouabain caused the cells to swell. The swelling was due to the continued entry of NaCl into the cells across their apical membranes. The resulting increase in solute content of the cells resulted in water flow into the cell by osmosis and subsequent cell swelling. The investigators utilized the ouabain-induced swelling to study the kinetics of NaCl entry into the cells across the apical membrane. Lowering the Na or Cl concentration in the solution facing the apical membrane to below 10 mM prevented the swelling. Replacement

of sodium by lithium also blocked solute entry. Bumetanide, a potent diuretic also prevented cell swelling by inhibiting the NaCl entry. The kinetics of the dependence of swelling on the Na and Cl concentrations of the bathing solution were identical, evidence that the kinetic constants were the same for both ions and that the transport process was tightly coupled. This supports the conclusion from other studies that transepithelial NaCl transport involves linked NaCl transport across the apical membrane. The importance of the new method, however, is that the transport process is being characterized much more accurately and completely than was previously possible.

(2) Cell volume regulation.

Fisher and Spring have monitored the electrical potential difference across the *Necturus* gallbladder epithelial cell membrane during osmotically induced changes in cell volume and subsequent volume regulation. The intracellular activities of chloride and potassium ions were also determined with ion specific microelectrodes. Thus, it was possible to calculate ionic fluxes across the apical membrane both during the initial osmotic change and subsequent volume regulation. Removal of bicarbonate from the bathing solutions prevented volume regulation. The requirement for bicarbonate is interpreted to indicate that H^+ and/or OH^- transport is required for the volume regulation. Also, the solute transport involved in volume regulation must differ basically from transepithelial transport, since the latter does not require bicarbonate. These interrelations are currently being investigated in more detail.

Red blood cell volume regulation.

Kregenow has been studying cell volume regulation using avian and amphibian erythrocytes as model systems. These cells correct their size in anisotonic media or in response to catecholamines by first selectively gaining or losing electrolytes, followed, in turn, by the osmosis of water. In *Amphiuma* the transporter that corrects the size of shrunken cells is a Na+/H+ exchanger that is inhibited by amiloride. The action of this drug on the *Amphiuma* red cell transporter resembles its action on epithelia with respect to kinetic characteristics, specificity and reversibility. Kregenow and Siebens are now studying this transport process in greater detail. They find that amiloride is a competitive inhibitor of the volume-regulatory Na uptake (K_I, $1\text{-}3 \times 10^{-6}$). The Na/H exchange rate varies directly with the magnitude of the initial cell shrinkage and with medium pH. Conditions during this time influence the rate of Na+/H+ exchange which in the extreme is 1000fold greater than control rate of Na+ exchange. There is a short delay between the initial cell shrinkage and the onset of volume regulation. The length of the delay, during which the mechanism is being triggered, is also influenced by the degree of cell shrinkage and medium pH. The investigators intend to characterize the system further and to attempt to isolate the carrier involved.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01217-06 KE

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Epithelial fluid transport and morphology

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

P.I.:	Kenneth R. Spring, DMD, Ph.D.	Physiologist	LKEM	NHLBI
Other:	Ann-Christin Ericson, Ph.D.	Visiting Fellow	LKEM	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

Electrolyte Transport

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Insitute, Bethesda, Md.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	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)

Epithelial cells swell when the Na-K pump is inhibited with ouabain. The swelling is due to NaCl entry into the cell across the apical membrane. The ionic dependence of this entry step was studied by replacing some of the Na or Cl in the medium bathing the apical cell membrane. NaCl entry into the cell was shown to be tightly coupled and carrier mediated.

PHS-6040
(Rev. 2-81)

Objectives

The primary goal of this investigation is elucidation of the mechanism of fluid absorption by epithelia. The methods employed represent a unique blend of optical and electrical techniques developed specifically for this investigation. We use an online, real time system for measuring the size and shape of the cells. Based on changes in cell volume we are able to determine the mechanism and rate of entry of NaCl into epithelial cells across the apical membrane.

Methods

The gallbladder of the amphibian Necturus maculosus is mounted in a chamber designed to allow the continuous perfusion of both surfaces of the tissue, measurement of epithelial electrical properties, variation in transepithelial hydrostatic pressure, and rapid alteration of perfusate composition. The chamber is placed on the stage of an inverted microscope. Position and focus of the microscope are monitored by observation of the preparation during measurements with the aid of a television camera. The video images are stored on a disc recorder and later analyzed by tracing cell outlines. Cell size and shape are determined from video records obtained during alteration in perfusate composition or other experimental manipulations.

Major Findings

The rate of NaCl entry into gallbladder epithelial cells was determined by measurement of the rate of cell swelling following inhibition of the cell's Na-K ATPase by application of ouabain. When the medium bathing the apical membrane contained 100 mM NaCl, the cell swelled at a rate of 4.3% min^{-1}. Swelling continued for about 10 minutes until a maximum volume of 1.45 times control had been achieved. The swelling was caused by NaCl entry into the cells across the apical membrane because it could be prevented by lowering the NaCl concentration of the bathing solution to 10 mM. Both Na and Cl were required in equal concentrations for cell swelling to occur. Replacement of Na or Cl by other ions prevented the swelling. The diuretic, Bumetanide, also inhibited NaCl entry into the cells and prevented cell swelling.

Proposed Course

We will test the effects of other inhibitors on the ouabain-induced swelling to verify that Na and Cl entry are tightly coupled by a carrier in the apical cell membrane. In addition we will attempt to inhibit K exit and observe the effect on the rate of cell swelling.

Publications

A-C Ericson and K.R. Spring. Mechanism of NaCl entry into Necturus Gallbladder Epithelium. Fed. Proc. 41(3): 360, 1981.

K.R. Spring, A. Hope, B-E Persson. Quantitative light microscopic studies of epithelial fluid transport, in Water Transport across Epithelia, ed. H.H. Ussing et al. Munksgaard, Copenhagen, 1981.

A.J. Coble, J.P. Leader, K.R. Spring. Microscope interferometry of Necturus gallbladder epithelium, in Conference on the paracellular pathway, ed. S.E. Bradely. J. Macy Foundation, N.Y. 1981.

B-E Persson and K.R. Spring. Gallbladder epithelial cell hydraulic water permeability and cell volume regulation. J. Gen. Physiol. 1981 (in press).

K.R. Spring. Quantitative light microscopy of epithelia, in First International Workshop on Developmental Renal Physiology, ed. A. Spitzer, Academic Press, N.Y. 1981.

A.M. Weinstein, J.L. Stephenson, K.R. Spring. The coupled transport of water, chapter 12, in Membrane Transport, ed. S.L. Bonting, J. de Pont, Elsevier, Amsterdam, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01224-05 KE

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Control of sodium and potassium transport in the nephron

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

P.I.:	Mark Knepper, M.D., Ph.D.	Staff Associate	LKEM	NHLBI
Other:	Maurice B. Burg, M.D.	Chief	LKEM	NHLBI
	J. Pisano, Ph.D.	Chief, PCS	LC	NHLBI
	David Proud, Ph.D.	Visiting Associate	LC	NHLBI

COOPERATING UNITS (if any)

Laboratory of Physiological Chemistry

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

Electrolyte Metabolism

INSTITUTE AND LOCATION

National Heart, Lung, Blood Institute, Bethesda, Md.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.30	1.0	0.30

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

As a prelimiary step in experiments to determine the role of the kallikrein-kinin system in the regulation of NaCl transport, experiments are being conducted to localize kallikrein along the nephron using radioimmunoassay.

PHS-6040
(Rev. 2-81)

Objectives

The major goal of this investigation is to define the role of the kallikrein-kinin system in the regulation of NaCl excretion. A preliminary objective is to identify which segments of the nephron have intracellular kallikrein and which respond to mineralocorticoid stimulus with a change in kallikrein content.

Methods

A radioimmunoassay technique developed in the Laboratory of Physiological Chemistry is used for the kallikrein localization studies. These studies are done on homogenized rat kidney slices, cut along the length of the corticomedullary axis. This will provide preliminary localization to specific regions of the kidney. Following this , renal tubules from specific sites along the nephron will be microdissected for kallikrein determination. Localization to specific nephron sites will allow the rational design of studies of kallikrein-kinin actions in isolated tubules.

Major Findings

In rat kidney slices, the highest kallikrein content (expressed per unit dry weight) is in the outer portion of the cortex (30 ng/mg dry weight). This value declines to 25-50% of the outer cortical value in the inner cortex. This pattern is consistent with the localization of kallikrein primarily in the cortical labyrinth since the fraction of the total cortical volume occupied by the labyrinth declines in the inner cortex by an amount similar to the change in kallikrein content. Alternatively, there could be differences in kallikrein content between superficial and juxtamedullary nephrons. A small, but detectable quantity of kallikrein was found throughout the medulla. This unexpected result should be investigated further.

Proposed Course

Additional slice studies will be conducted to determine whether mineralocorticoids induce a change in tissue kallikrein content. Following this, kallikrein will be determined in pooled nephron segments microdissected from rat kidneys.

Significance

Localization of kallikrein in the kidney is a necessary preliminary to rational design of transport experiments in isolated nephron segments. Such transport experiments may shed some light on the role of the kallikrein-kinin system in the regulation of renal NaCl excretion.

Publications

L.C. Garg, M.A. Knepper and M.B. Burg. Mineralocorticoid effects on Na-K-ATPase in individual nephron segments. Am. J. Physiol. (in press).

M.A. Knepper and M.B. Burg. Increased fluid absorption and cell volume in isolated rabbit proximal straight tubules following *in vivo* DOCA administration. Am. J. Physiol. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01237-03 KE

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Hormonal control of transport in a line of toad kidney epithelial cells in culture

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

P.I.	J.S. Handler, M.D.	Head, Section on Membrane Metabolism	LKEM	NHLBI
Others:	P. Popowicz, M.D.	Guest Worker	LKEM	NHLBI
	A.S.Preston, B.S.	Chemist	LKEM	NHLBI
	R. Steele, Ph.D.		LTD	NHLBI

COOPERATING UNITS (if any)

Laboratory of Technical Development, NHLBI

LAB/BRANCH

Laboratory of Kidney & Electrolyte Metabolism

SECTION

Membrane Metabolism

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, Md.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.8	2.8	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)

Toad kidney cells derived from Xenopus laevis form a monolayer epithelium of high electrical resistance. Aldosterone occupancy at specific nuclear binding sites correlates in a linear manner with the stimulation of sodium transport and there is increased ouabain binding to membrane preparations. Cyclic AMP analogs also stimulate sodium transport.

PHS-6040
(Rev. 2-81)

Objectives

Because of the limitations of established techniques for studying epithelial transport and its control, this project is intended to develop appropriate cell culture methods and material and to study transport mechanisms and their control in the cultured epithelia.

Methods

See last year's report and the annual report of Dr. R. Steele, LTD, NHLBI.

Major Findings

Previous work in this project has demonstrated hormone responsive transepithelial transport by epithelia formed in culture. Cells of the continuous line designated A-6, derived from the kidney of Xenopus laevis, form epithelia with a high transepithelial electrical resistance and active transepithelial sodium transport that can be stimulated by aldosterone. Although we have been unable to detect hormone sensitive adenylate cyclase activity, cAMP stimulates sodium transport by A-6 epithelia. We have assumed that the cells have lost their ability to make cAMP in response to hormones, but that the transport response to the intracellular mediator (cAMP) is a remnant of the action of some hormone that stimulates sodium transport in the kidney of X. laevis. Originally, transport studies were performed on collagen coated nucleopore filters (filter bottom cups) that rested on the bottom of a petri dish. A-6 cells were later grown on membranes formed of collagen (no filter), a technique that requires that the collagen membrane be spaced off the bottom of the petri dish. Uner those circumstances the epithelial cells had active cilia, a differentiated property that was not observed previously. We have now found that A6 epithelia grown on collagen membranes or on filters spaced off the bottom of the petri dish not only form cilia, but have a sodium transport response to vasopressin as well as to cAMP. We conclude that epithelia, which in situ obtain nutrition via their basal (blood) surface, are more differentiated under culture conditions that allow nutrient exchange at that cell surface. Such nutrient exchange apparently was very limited in standard culture conditions on dishes and in our original version of filter bottom cups. Further study of A-6 epithelia in culture has revealed significant instability of potential difference and short-circuit current after the solutions bathing the epithelium are replaced. We have identified this as an effect of mixing rather than removal or addition of a specific material. This response to mixing can be avoided by continuously mixing the solutions bathing the epithelia.

In preliminary studies, we have found that norepinephrine stimulates sodium transport (short-circuit) current across A-6 epithelia grown on collagen filters.

A similar response is known to occur in frog kidney.

Significance

The improved culture conditions should be of great value in obtaining cultured epithelia with differentiated properties of interest. Control of the effect of mixing is important since the A-6 epithelia have now been shown to have a sodium transport response to four hormones (vasopressin, aldosterone, norepinephine, and insulin (C. Watlington, personal communication)) each initially acting on the cell by a different mechanism.

Proposed Course

The culture conditions are being modified to eliminate the effect of mixing on sodium transport. The stimulation of transport by norepinephrine will be characterized by the use of specific adrenergic blocking agents.

Publications

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01241-02 KE

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Ionic composition of Necturus gallbladder cells

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

P.I.:	Kenneth R. Spring, DMD, Ph.D.	Physiologist	LKEM	NHLBI
Other:	Richard S. Fisher, Ph.D.	Staff Fellow	LKEM	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Kidney and Electrolyte Metabolism

SECTION
Electrolyte Transport

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, Bethesda, Md.

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)

Intracellular ionic activities of potassium and chloride are being measured in Necturus gallbladder epithelial cells undergoing osmotically induced volume changes.

PHS-6040
(Rev. 2-81)

Objectives

Necturus gallbladder epithelial cells undergo volume changes when exposed to solutions whose osmolality differs from control solutions. Osmotically induced cell shrinkage or swelling is followed by a rapid readjustment of cell volume back toward the control volume. Such readjustment is termed "volume regulation" and in other tissues results from the flow of solutes into or out of the cell. The object of these experiments is the identification and quantitation of the ion movements responsible for the observed volume regulation.

Methods

Ion sensitive microelectrodes have been constructed. The electrodes are made from microfiber glass capillaries drawn to a tip of diameter less than 1 μm. The electrodes are siliconized by dipping the tip into trichloromethylsilane for a few seconds and then heating them. The electrodes are stored unfilled until just before use when they are filled from the back end with liquid ion exchange resin. The ion exchangers used in these experiments were sensitive to Cl^- or K^+. Since the ion sensitive electrode has a very high electrical resistance (10^{10} ohms) it exhibits slow response time and great sensitivity to electrical interferences. It is connected to a high impedance electrometer whose output is displayed on a digital voltmeter, interfaced to a small computer. Determination of the end point of electrode drift and slope sensitivity to K^+, or Cl^- is then made objectively by this computer system. A chamber has been designed which permits rapid exchange of the solutions bathing either surface of the gallbladder epithelium.

Major Findings

Intracellular chloride and potassium activities have been determined during changes in the osmolality of the solution bathing the mucosal surface of the Necturus gallbladder. Mucosal hypertonicity created by the addition of mannitol to the mucosal perfusate caused an increase in intracellular chloride activity. This increased activity was diminished in magnitude when the perfusion solutions contained bicarbonate ions. Complete elimination of bathing solution bicarbonate abolished cell volume regulation. This shows that cell volume regulation was dependent on the presence of bicarbonate in the bathing solution and that the initial transient cell volume decrease following extra cellular hypertonicity was due to water flow out of the cell across the cell membranes without large losses or gains of intracellular ions.

Proposed Course

We will continue to investigate the changes in intracellular ionic composition caused by alterations in solution osmolality and resultant volume regulation. We will pay particular attention to alterations in intracellular sodium activity and to the interrelationship between sodium and hydrogen ions.

Publications

T. Shindo and K.R. Spring. Chloride movement across the basolateral membrane of proximal tubule cell. J. Memb. Biol. 58: 3542, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01242-02 KE

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Functional characteristics of the connecting tubule

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

P.I.:	Antonio Almeida, Ph.D.	Visiting Fellow	LKEM	NHLBI
Other:	Maurice B. Burg, M.D.	Chief	LKEM	NHLBI

COOPERATING UNITS (if any)

Computer Systems Laboratory, DCRT

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

Renal Mechanisms

INSTITUTE AND LOCATION

National Heart, Lung, Blood Institute, Bethesda, Md.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.10	1.0	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 objective of the project is to delineate some of the functional characteristics of the connecting tubule. The transepithelial electrical resistance and voltage were measured and the unidirectional sodium fluxes were determined isotopically. The influence of the B-agonist Isoproterenol on the sodium and chloride fluxes is being tested.

PHS-6040
(Rev. 2-81)

Objectives

Recent morphological and biochemical studies have shown that the distal renal tubule is composed of three different segments: the distal convoluted tubule (DCT), the connecting tubule (CNT) and the collecting tubule. Since the DCT and CMT are short segments and difficult to identify and to dissect, their functional characteristics are still poorly known. It was demonstrated that both segments have a negative transtubular electrical potential difference (PD), a low water permeability and that the connecting tubule shows a drop in the PD in the presence of Isoproterenol. Biochemical studies from this laboratory showed also a high level of activity for the sodium-potassium ATPase in this segment, suggesting a high level of sodium transport.

The objective of the project is to study some of the functional characteristics of the CNT by determining its electrical resistance and voltage, measuring its sodium and chloride transepithelial fluxes and the influence of the B-agonist Isoproterenol on those fluxes.

Methods

Segments of CNT obtained from rabbit kidneys were dissected and perfused "in vitro". Techniques developed in this laboratory and described previously were employed in the electrical and ionic flux measurements. A computer system was developed by the Computer System Laboratory (NIH) to help and improve the electrical resistance measurements.

Major Findings

(1) The electrical measurements showed a transepithelial potential difference between 20 and 40 mV, lumen negative, extremely sensitive to changes in the perfusion pressure; the mean transepithelial electrical resistance measured in 7 segments was 31 ohm cm^2.

(2) The sodium lumen to bath flux was 33.6 pEq/cm sec. while the sodium bath to lumen flux was 11.0 pEq/cm sec. The comparison of the two series give a net sodium efflux of 20 pEq/cm sec which is a relatively high rate of sodium transport. The lumen to bath sodium flux is not affected by the addition of Isoproterenol to the bath.

Proposed Course

Experiments are being done to determine the transepithelial flux of chloride and the possible influence of Isoproterenol on it. In another series, the transepithelial potassium flux will be measured.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01243-02 KE

PERIOD COVERED
October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)

D-glucose transport across the renal proximal tubule brush border membrane

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

P.I:	R. James Turner, Ph.D.	Visiting Associate	LKEM	NHLBI
Other:	A. Moran, Ph.D,	Visiting Associate	LKEM	NHLBI
	Janet N. George	Chemist	LKEM	NHLBI
	Douglas Tang	Chemist	LKEM	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute

TOTAL MANYEARS: 1.4	PROFESSIONAL: 0.6	OTHER: 0.8

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The kinetics, specificity and stoichiometry of D-glucose transport across the renal proximal tubule brush border membrane are being studied in vesicle preparations.

PHS-6040
(Rev. 2-81)

Objectives

The continuing goal of this project is to elucidate on both physiological and molecular levels the mechanism by which D-glucose is reabsorbed from the urine in the proximal tubule. We are presently concentrating on the kinetics, specificity and stoichiometry of transport in isolated brush border membrane vesicles.

Methods

Methods for preparing purified brush border membrane (BBM) vesicles and for carrying out transport and binding studies on them have been discussed in the 1979-80 Annual Report.

Major Findings

In the 1979-80 Annual Report we described two renal brush border membrane (BBM) vesicle preparations, one from the outer cortex and the other from the outer medulla. We reported there early experiments on these preparations which indicated that they exhibited markedly different sodium-dependent D-glucose transport properties. A low affinity, high capacity glucose transport system was found in the outer cortical preparation while a low capacity, high affinity system was found in the outer medulla. Since the outer cortical and outer medullary preparations contain predominatly BBM from the early and late proximal tubule, respectively, these results are strongly suggestive of D-glucose transport heterogeneity along the length of the proximal tubule BBM.

Over the past year we have gone on to study the glucose transport properties of these two vesicle preparations in detail in order to better understand the physiological implications of this heterogeneity.

In the outer cortical preparation we find that the sodium-dependent component of D-glucose flux is characterized by a site with an apparemt K_m of approximately 3.4 mM and a V_{max} of approximately 100 nmoles/min/mg protein as measured under zero trans conditions at 60 mM NaCl and 28°C. By contrast, in the outer medullary preparation this component of flux is characterized by a system with an apparent K_m of 0.6 mM and a V_{max} of 35 nmoles/min/mg protein. The ability of various monosaccarides and inhibitors to affect D-glucose flux into the two preparations is somewhat different indicating differences in specificity properties. For example, we find that D-galactose is a much better substrate for the outer medullary system than for the outer cortical one. Also, glucose uptake by outer cortical vesicles is significantly more sensitive to inhibition by the compound phlorizin (the K_I for phlorizin inhibition of D-glucose flux is approximately 10 μM in the outer cortex vs. 30 μM in the outer medulla).

In more detailed kinetic studies of the interactions of phlorizin, glucose and sodium with their respective binding sites on the outer cortical transporter we have established that the stoichiometry of the transporter is 1:1:1 (phlorizin:glucose:sodium) ie., that one phlorizin molecule competes with one glucose molecule for a site on the transporter and that this "substrate site" is associated with a single sodium binding site. Preliminary results of similar experiments on the outer medullary transporter indicate a sodium:glucose stoichiometry which is greater than one.

Proposed Course

Continued detailed study of the glucose transport and phlorizin binding properties of the outer cortical and outer medullary sites is planned.

Publications

R. James Turner and M. Silverman. Interaction of phlorizin and sodium with the renal brush border membrane D-glucose- transporter: stoichimetry and order of binding. J. Membrane Biol. 58: 43-55 (1981).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01244-02 KE

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Study of transport by vesicles prepared from cultured epithelia

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

P.I.:	Arie Moran, Ph.D.	Visiting Associate	LKEM	NHLBI
Other:	R. James Turner, Ph.D.	Visiting Associate	LKEM	NHLBI
	J. S. Handler, M.D.	Investigator	LKEM	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Kidney and Electrolyte Metabolism

SECTION
Membrane Metabolism

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, Bethesda, Md.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

We are developing methods for the preparation of plasma membrane vesicles from cultured epithelial cells. The vesicles will be used to study epithelial transport mechanisms.

PHS-6040
(Rev. 2-81)

Objectives

The objective of this project is to study membrane transport processes by preparing vesicles from apical or basolateral plasma membrane of cultured epithelial cells known from previous studies to exhibit to particular transport systems of interest.

Methods

Epithelia formed by cells of the line designated LLC-PK_1 derived from pig kidney have been used. They were chosen because they are known to transport glucose and phosphate by a sodium-coupled mechanism similar to that of proximal renal tubule. Vesicles are prepared by a method that combines hypotonic shock, Virtis homogenization, and precipitation by high calcium. Enrichment of apical plasma membrane is evaluated with standard enzyme assays (see last years report). To study the uptake (transport) of glucose and its analogs and the effect of the specific inhibitor phlorizin, standard methods have been modified.

Major Findings

The vesicles are suitable for studying transport properties of the apical plasma membrane. They are highly enriched in an apical membrane marker (20 x activity in whole homogenate) and are not enriched (0.5-2x) in markers of other membranes. Mean vesicle volume is about 1.3 ul per mg. protein.

Sugar transport by the vesicles has many characteristics seen in vesicles prepared from apical plasma membranes of mammalian proximal tubules. In the presence of a 100 mM sodium gradient, the uptake of glucose and of methyl glucoside is characterized by a transient overshoot of intravesicular sugar concentration above its equilibrium value. This provides evidence of sodium-coupled sugar transport. The transport is inhibited by phlorizin. Phlorizin binding to vesicles is sodium dependent and is competitively inhibited by glucose and by alpha methyl glucoside. The specificity of the transporter for different monosaccharides resembles that found in the proximal tubule of mammalian kidney; glucose, -methyl glucose, and galactose share the transporter while 2-deoxyglucose, mannose and fructose do not. The affinity of the transporter for substrate is like that found in vesicles prepared from proximal tubules (1-3 mM), but the capacity is much lower. The low number of transport sites is also reflected in low maximal binding of phlorizin.

Proposed Course

Glucose uptake will be studies as a function of sodium concentration to estimate the sugar-sodium stochiometry of the transporter. After the transporter is well characterized in vesicles prepared from cells grown in the usual fashion, growth conditions will be changed to alter epithelial transport. The effect of the changed growth

conditions on the transporter in vesicles prepared from the altered epithelia will then be examined.

Publications

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01245-02 KE

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Amiloride-sensitive volume regulation in Amphiuma red cells

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

P.I.:	Arthur W. Siebens	Biologist	LKEM NHLBI
Other:	Floyd M. Kregenow, M.D.,	Sr. Inves.	LKEM NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, Md.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.4	1.4	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Amphiuma red cells incubated in hypertonic media regulate their volume by using an amiloride-senstive Na transport mechanism. Under certain circumstances, Na transport increases over 1000-fold relative to control conditions. Under all conditions, this increased Na entry is regulated. Kinetic analysis indicates that amiloride behaves as a competitive inhibitor or Na transport in these cells.

PHS-6040
(Rev. 2-81)

Objectives

Red cells from a number of animals can regulate their volume in anisotonic media. These cells utilize highly specific transport mechanisms to control the gain and loss of cellular electrolytes. The changes in electrolyte content result in osmotic water movements which bring about alterations in cell volume.

In previous annual reports we have shown that the giant erythrocytes of the salamander *Amphiuma* were able to regulate their volume when shrunken osmotically in hypertonic media. This volume regulatory increase (VRI) was not affected by inhibiting the Na/K pump with ouabain. Cells enlarged by increasing their cellular Na content so that the final Na content was several-fold greater than that of control cells. An increase in cell chloride was also observed. Amiloride completely inhibited the Na gain, thus preventing cells from regulating their volume in hypertonic media. Since a number of epithelia have amiloride-sensitive Na transport mechanisms, we identified characteristics shared by the *Amphiuma* red cell Na transporter and amiloride-sensitive transporters in epithelia. Among the shared characteristics were: (1) Amiloride inhibition is readily reversible (2) the amiloride sensitive Na movements are a saturable function of external Na (3) Lithium can substitute for Na but K cannot. An apparent difference, however, between the *Amphiuma* red cell Na transporter and many of the amiloride sensitive transporters described in epithelia was that the *Amphiuma* transporter acted as an electrically neutral Na^+/H^+ exchanger while most amiloride-sensitive transporters in epithelia are believed to be electrically conductive Na channels.

Our objectives in the studies reported here were as follows:

(1) to investigate the mechanism whereby amiloride inhibits Na transport (2) to determine whether the membrane potential changes during the VRI reponse (3) to identify factors affecting the magnitude of the Na uptake response in an effort to further understand this transport process.

Methods

The methods utilized in these experiments have been described in previous annual reports.

Major Findings

(1) 5×10^{-6} M amiloride increased the K_m for Na influx during VRI from about 30 mM to about 100 mM Na while the V_{max} remained unchanged.

Thus, amiloride appears to act as a competitive inhibitor of volume-stimulated Na uptake. It follows that amiloride blocks a greater percentage of the Na influx when the medium Na is low than when medium Na is high. The K_I for amiloride is $1\text{-}3 \times 10^{-6}$ M.

(2) A technique devised by Stoner and Kregenow for directly measuring the membrane potential of _Amphiuma_ erythrocytes has established that the ratio of intracellular chloride to extracellular chloride concentration is a valid indicator of the membrane potential under most circumstances. Chloride ratios did not change substantially during the VRI response, suggesting that electrically silent Na/H^+ coupled movements are involved. Direct microelectrode impalements of _Amphiuma_ red blood cells during VRI confirmed the absence of the large changes in P.D. However, the chloride ratios did change slightly by 30 minutes consistent with a 5 mV decrease in the membrane potential by that time.

(3) Several pertubations were shown to change the rate of amiloride sensitive Na entry.

(a) Increasing the degree of cell shrinkage by increasing the medium osmolarity at constant medium Na results in an increase in net Na uptake. Maximal rates of the Na uptake are obtained when the medium osmolarity is about twice plasma osmolarity.

(b) Increasing medium pH also increases Na uptake. Changing the medium pH from the usual 7.7 to 8.1 increases the Na uptake over two fold; decreasing the medium pH to 7.1 substantially decreases Na uptake.

(c) The Na uptake also varies as a function of time, becoming maximal in about 15 minutes when the medium is 1.5 x plasma osmolarity. This effect of time is also a function of cell shrinkage. Increasing the degree of shrinkage by increasing the medium osmolarity reduces the time required for the maximal response.

(d) Treating cells with amiloride in hypertonic media and then washing off the amiloride causes Na uptake to exceed that of untreated cells of the same time. The magnitude of the Na uptake in treated cells is a function of how long the cells have been exposed to amiloride. Under optimal conitions Na uptake can be increased over 1000-fold relative to the uptake of cells in isotonic media. With a Na uptake this rapid, treated cells complete the VRI response in as little as 15 minutes (3-4 fold faster than normal). There is similar enhancement of the VRI response when shrunken cells are exposed to a Na-free hypertonic medium and thereby prevented from enlarging. Subsequent exposure to a Na-containing hypertonic medium produces the enhanced response. In general, cells take up Na, albeit at different rates, until they approach their original volume.

(e) After completion of the VRI response the unidirectional Na influx remains elevated for at least an hour in the absence of changes in net Na content. Under these conditions, Na transport is consistent with a Na/Na exchanger.

Significance

Intracellular Na concentration, acting through a negative feedback mechanism is purported to regulate amiloride-sensitive Na entry in epithelia. Whether intracellular Na is indeed the important variable remains unclear, in light of recent evidence which suggests that both volume and intracellular Na decrease. Our studies suggest that some factor related to cell volume and not intracellular Na concentration regulates amiloride-sensitive Na entry in *Amphiuma* red cells. We suggest that the same may be true in epithelia and that the *Amphiuma* red cell model of Na entry responding to cell volume may be applicable to amiloride-sensitive transport in epithelia as well.

Proposed Course

We plan to examine the mechanism by which amiloride-sensitive Na transport is regulated in *Amphiuma* red cells. Special emphasis will be placed on elucidating how transporters which have been activated during the VRI response become inactivated when the VRI response is completed. In addition we plan to alter cell Na content, cell volume and hydrogen ion gradients selectively by using ionophores for Na and H^+. By combining these techniques with osmotic perturbations, we hope to clarify the role cell volume and hydrogen ion gradients play in the regulation of amiloride-sensitive Na movements in these cells. The possible role of Ca in *Amphiuma* red cell volume regulation will also be investigated.

Publications

Kregenow, F.M. 1981. Osmoregulatory salt transporting mechanisms: Control of cell volume in anisotonic media. Ann. Rev. Physiol. 43:493-505.

Stoner, L.C., and F. M. Kregenow 1980. A single cell technique for the measurement of membrane potential, menbrane conductance, and the efflux of rapidly penetrating solutes in *Amphiuma* erythrocytes. J. Gen. Physiol. 76:455-478.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01246-01 KE

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Culture and Function Study of Epithelial Kidney Cells

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

P.I.:	Maurice B. Burg, M.D.	Chief	LKEM	NHLBI
Other:	Sarah Sariban-Sohraby, M.D.	Guest Worker	LKEM	NHLBI
	Nordica Green, B.S.	Chemist	LKEM	NHLBI
	Joseph Handler, M.D.	Investigator	LKEM	NHLBI
	Roderic Steele, M.D.	Investigator	LTD	NHLBI

COOPERATING UNITS (if any)

Laboratory of Technical Development, NHLBI

LAB/BRANCH
Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, Bethesda, Md.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.6	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)
Epithelial cells derived from the medullary thick ascending limb of rabbit kidney are studied in primary culture; they show a transepithelial voltage characteristic of the original tubule and the same response to furosemide; this indicates the presence of a differentiated cell function in the culture.

PHS-6040
(Rev. 2-81)

Objective

The goal of this research is to elucidate the mechanisms of the NaCl transport in the medullary thick ascending limb cells in culture. The first step was to be able to grow the cells (see previous annual report); the second has been to identify a cellular function.

Methods

The cells grow on a collagen membrane designed so that the cells are in contact with the growth medium both at their basal and apical side; the transepithelial voltage is measured between apical and basal solutions as described previously by Dr. Steele.

Major Findings

The cells develop a transepithelial voltage after 10 days in culture; this voltage is oriented apical side positive and decreases significantly when furosemide is added to the mucosal side of the cell but does not respond to amiloride or vasopressin. Thus, the cells in culture show an electrical potential that has the same characteristics as those observed in the intact perfused medullary thick ascending limb. This indicates the maintenance of a differentiated cell function.

Proposed Course

We are trying to establish a cell line from the primary culture in order to get enough material to study the mechanisms of the Na Cl transport. Once lines are established they will be studied by chemical and radioisotope methods using confluent cultures and vesicles made from the plasma membranes of the cells.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 HL 01247-01 KE

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Urea transport in the kidney

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

P.I.:	Mark Knepper, M.D., Ph.D.	Staff Associate	LKEM	NHLBI
Other:	G. Vurek, Ph.D.	Sr. Investigator	LTD	NHLBI

COOPERATING UNITS (if any)

Laboratory of Technical Development, NHLBI

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

Electrolyte Transport

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, Md.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	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)

Urea transport by isolated, perfused rabbit renal tubule segments is studied. The cortical thick ascending limb of Henle has been found to have a significant permeability of urea. This may allow recycling of urea between the thick ascending limb and the proximal straight tubules within the cortical medullary rays.

PHS-6040
(Rev. 2-81)

Objectives

The chief long term goal of this project is to understand the role that urea plays in urinary concentration and dilution. An intermediate goal is to determine the extent and mechanism of urea transport in each nephron segment.

Methods

Urea transport in single rabbit nephron segments is being studied using a method for perfusing isolated tubules *in vitro* developed in this laboratory. Urea is measured using a ultra-micro colorimetric method developed by Dr. G. Vurek. ^{14}C-labelled urea is used when an isotopic label is required. The change in urea concentration in the luminal fluid is determined with and without a chemical gradient of urea across the epithelium. This allows assessment of both the passive permeability properties and active transport capacity of the nephron segment.

In addition, a method has been developed for the measurement of osmolality in the tissue fluid of intact kidney slices using vapor pressure osmometry. This method allows greater precision and accuracy than previous techniques. Tissue urea and electrolyte content can be measured in the same slices.

Major Findings

Urea Transport by Cortical Thick Ascending Limbs of Rabbit

Condition (n)	Bath Urea Conc(nm)	Perfused Urea Conc (nm)	Perfusion Rate (nl/min)	Urea Flux (pmol/cm/sec)	Voltage (mv)
I(4)	0	5	3.4±0.7	0.67±0.18	3.3±0.7
II(4)	5	0	4.2±0.3	-0.56±0.11	4.4±1.2
III(3)	5	5	2.7±0.6	0.06±0.08	8.1±1.5
IV(3)	0	20	5.3±1.6	1.98±0.51	3.0±0.9

These results show that the cortical thick ascending limb has a significant permeability to urea. Evidence for active urea transport has not been found.

Tissue slice studies in rabbits reveal that tissue fluid is close to isotonic in the outer cortex and slightly hypotonic in the midcortex. The tissue fluid urea concentration is approximately twice that of plasma throughout the cortex.

Significance

The mechanism for urea transfer into the inner medulla is not totally understood. Berliner proposed that urea enters the inner medullary interstitium from the collecting ducts and is trapped by the countercurrent exchange system. Subsequently, Stephenson and Kokko and Rector have proposed that NaCl can be concentrated in the inner medulla using energy derived from the dissipation of the high urea concentration in the inner medullary interstitium. While these ideas are theoretically sound, quantitative models of medullary transport function raise the question of whether enough urea can be absorbed from the inner medullary collecting ducts to account for the urea and NaCl gradients seen in tissue slice studies. Thus we are led to consider an alternative source of urea supply to the inner medulla, via the descending limbs. Large concentrations and mass flow rates of urea could result in the descending limb if urea is recycled by the loops of Henle. For recycling to occur, a mechanism must exist for movement of urea from ascending limbs to descending limbs at some level. One region where this may occur is the cortical medullary rays where evidence already exists for urea entry into the proximal straight tubules by an active secretory process. The structure of the medullary rays seem to be particularly suited for transfer of substances from cortical thick ascending limbs to proximal straight tubules. The proximal straight tubules tightly enclose the core of the ray where the cortical thick ascending limbs run. Thus, urea leaving the cortical thick ascending limbs would have to pass through or past the proximal straight tubules to enter the cortical labyrinth, a region of rich blood supply. It has not been known until now whether the cortical thick ascending limb is capable of urea absorption either actively or passively. Evidence is presented above that the urea permeability of the cortical thick ascending limb is adequate to allow significant passive absorption. This finding is consistent with the possibility of recycling of urea in the loops of Henle.

Proposed Course

Experiments will be conducted to further characterize the urea transport mechanism in the thick ascending limb. Since, a significant urea permeability is seen in an epithelium that is essentially water impermeable, it seems reasonable to propose that a relatively specific pathway exists for urea transport. Studies of inhibition with urea analogs, inhibition of transport by phloretin (which inteferes with urea transport in other tissues), and saturation of transport at higher urea concentrations may help to clarify this issue. Further studies will be conducted with other segments that may exhibit physiologically important urea transport, particularly the cortical arcades, proximal straight tubules, descending limbs, and medullary thick ascending limbs. Other techniques that will be utilized as needed are tissue slice analysis and mathematical modelling.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01249-01 KE

PERIOD COVERED

October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)

Isolation and study of brush border membrane transport proteins

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

P.I.:	R. James Turner, Ph.D.	Visiting Associate	LKEM	NHLBI
Other:	Janet N. George	Chemist	LKEM	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, Md.

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)

Isolation of the renal brush border membrane D-glucose transporter is being attempted by conventional protein solubilization and purification techniques and by the development of specific monoclonal antibodies against the transporter.

PHS-6040
(Rev. 2-81)

Objectives

The immediate goal of the project is to develop methods for isolating brush border membrane transport proteins in purified form. Our long term objective is to study these isolated proteins using a number of established biophysical and biochemical techniques in order to gain information about their structure and mechanism of action. Two parallel and complimentary approaches are being employed, (a) to attempt to extract and (partially) purify the transport-related proteins using conventional protein separation methods and (b) to attempt to grow monoclonal antibodies against the proteins of interest. At present we are concentrating on the outer cortical renal brush border membrane D-glucose transporter since this protein may be followed throughout detergent extraction procedures by its ability to bind the compound phlorizin.

Methods and Major Findings

(a) Solubilization studies

A number of detergents are currently being evaluated on the basis of their ability to solubilize the glucose transporter leaving its phlorizin binding capacity intact. Outer cortical brush border membrane (BBM) vesicles are being used as the starting materials for these solubilization studies. To date a simple "solid-phase" binding assay has been employed. Solubilized proteins are bound to the adsorbant surface of a plastic microtiter plate, washed to remove detergent and incubated with (^{3}H) -phlorizin. On the basis of this assay the ionic detergent cholic acid has been selected for more detailed study.

(b) Monoclonal antibodies

We have established protocols for immunizing BALB/c mice with outer cortical brush border membrane vesicles and have set up a solid phase radioimmune assay to monitor their response. High levels of anti-BBM antibodies are typically found in preimmunized mice after intraperitoneal boosts of BBM vesicles.

We have worked out techniques for fusing immune spleen cells with myeloma cells (Sp 2/0) and for cloning fusion products of interest by adapting and modifying the techniques of other investigators. To date a successful fusion has been carried out and five cell lines have been selected for future study. These lines were chosen on the basis of the apparent ability of the antibodies they produce to inhibit or stimulate phlorizin binding to BBM vesicles.

Proposed Course

Monoclonal antibody studies will be continued with the aim of producing a series of specific anti-glucose-transporter antibodies A number of protein solubilization and purification techniques will

be attempted in order to obtain more purified preparations of the transporter which can be used in turn in the monoclonal antibody studies as antigens for injection and as a more sensitive means of detection of antibodies.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01250-01 KE

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Acidification and Bicarbonate Transport by Renal Tubules

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

P.I.:	James Atkins, M.D., Ph.D.	Guest Worker	LKEM	NHLBI
Other:	Maurice B. Burg, M.D.	Chief	LKEM	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

Electrolyte Transport

INSTITUTE AND LOCATION

National Heart, Lung and Blood Institute, Bethesda, Md.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.10	1.0	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)

Maximum transepithelial pH gradient is determined in isolated perfused proximal straight tubules and the determinants for this gradient are assessed by various ion substitutions.

PHS-6040
(Rev. 2-81)

Objectives

The purpose of this study is to examine the factors controlling acidification in the kidney. In particular this study focuses on one measure of acidification, the maximum pH gradient that the tubule can maintain. In previous studies in this lab, measuring bicarbonate reabsorption, the proximal tububle has been shown to have a rapid hydrogen ion secretory rate but this segment is normally only capable of creating a small transepithelial pH gradient of about 0.5 pH units. This study is attempting to delineate the factors that determine this gradient.

Methods

Proximal straight tubules are dissected and perfused as previously described. pH of the collected fluid is measured in the collecting pipette using a micro glass pH electrode. The design of this pipette system is adequate to measure pH without artifact from diffusional loss of CO_2 before the measurement. pH is determined at slow perfusion rates and accepted as a steady state value only if it is unchanged with a doubling of the collection rate and/or is the same with perfusates of lower and higher pH. The effect of various ion substitutions on this value is determined.

Major Findings

These studies are still in progress. With a bath pH of 7.4 the tubule can maintain an average minimum pH of about 6.9. This value is the same in nominally bicarbonate-free solutions, suggesting that bicarbonate back-leak may not be the major determinant of the transepithelial pH gradient. The pH gradient in sodium and bicarbonate-free solutions is much less (lumen pH of 7.3). This supports the theory of a Na-H exchanger in this segment.

Proposed Course

Further studies are needed in the proximal tubule to define what other mechanisms are involved in acidification in this segment. Similar studies are planned in the cortical collecting tubule where a large transepithelial gradient is expected.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01251-01 KE

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Non-invasive studies of renal cellular energetics

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

P.I.:	Robert S. Balaban, Ph.D.	Staff Fellow	LKEM	NHLBI
Other:	Jack Orloff, M.D.	Director	IR	NHLBI
	John Bader, M.D.	Head	CGS	NCI
	Richard Knazek, M.D.	Investigator	CIP	NCI

COOPERATING UNITS (if any)

National Cancer Institute

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, Bethesda, Md.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.30	1.00	0.30

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Renal cellular metabolic intermediates are monitored non-invasively using optical spectroscopy, nuclear magnetic resonance spectroscopy or selective electrode to determine the metabolic control sites within the intact cell. Specifically, the coupling mechanism between the rate of metabolic energy conversion and active ion transport in the kidney is being investigated.

PHS-6040
(Rev. 2-81)

Objectives

The primary goal of this investigation is to establish the control sites which balance the rate of energy conversion with the rate of energy utilization by active ion transport in kidney cells. Using non-invasive techniques coupled with classical *in vitro* procedures we are investigating how the substrate concentration and kinetic parameters change with energy utilization. Based on these results, it is hoped that a clearer understanding will emerge of how the metablic processes are controlled within renal epithelial cells.

Methods

Six different preparations of cells whose transport function have been characterized are being used or developed: (1) tubule suspensions from different regions of the kidney. (2) isolated perfused and nonperfused tubule segments. (3) *in vivo* kidney (4) cultured renal cell lines (in collaboration with Dr. J. Handler and Dr. R. Knazek) (5) transformed cell lines (in collaboration with Dr. I. Bader) (6) isolated renal mitochondria. The techniques employed are optical spectroscopy and fluorescence to detect the redox state of the mitochondrial cytochromes and pyridine nucleotides. ^{31}PNMR to detect intracellular ATP, ADP, Pi and pH. ^{13}CNMR is also being developed. Oxygen, carbon dioxide and glucose sensitive electrodes are used to determine the rate of production or consumption of these metabolites. In most cases, related variables are measured simultaneously to establish the relationships existing between them. Chambers have been designed to monitor oxygen consumption, CO_2 production, cytochrome and pyridine nucleotide redox state, metabolite concentrations (NMR), and glucose.

Major Findings

In the few months of this project, the emphasis has been on development of techniques outlined in methods.

Proposed Course

Utilizing the specific experimental advantages of the preparations listed, experiments will be conducted to investigate the steady state concentration and kinetic properties of key intracellular metabolites during changes in the rate of energy conversion (i.e. oxygen consumption, glycolysis and CO_2 production) and energy utilization (i.e. active transport). These studies will establish how the metabolites respond to change in work output and may elucidate the metabolic control sites within the intact cells.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01252-01 KE

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Solute and water pathways in epithelial barriers

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

P.I.:	Jacques Chevalier, Ph.D.	Guest Worker	LKEM	NHLBI
Other:	Joseph Handler, M.D.	Head, Section on Membrane Metabolism	LKEM	NHLBI
	Pedro Pinto da Silva, Ph.D.	Head, Section on Biomembrane	LPP	NCI

COOPERATING UNITS (if any)

Laboratory of Pathophysiology, Section of Biomembranes, NCI

LAB/BRANCH

Laboratory of Kidney and Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.20	1.0	0.20

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The effects of antidiuretic hormone and cyclic AMP on intramembranous particle aggregates in $LLC\text{-}PK_3$ cells was studied by freeze fracture electron microscopy.

PHS-6040
(Rev. 2-81)

Objectives

The goals were, as follows:

(1) We demonstrated previously that the water permeability response to ADH is associated with the appearance of aggregates of intramembranous particles in the apical plasma membrane of ADH-sensitive cells. There is considerable evidence linking these particle aggregates to the water permeability response. Recently, epithelial cells derived from a variety of organs sensitive to the antidiuretic hormone (ADH) (i.e., mammalian kidney and amphibian bladder) have been maintained in culture. Some of these cell lines have adenylate cyclase sensitive to ADH, others have sodium transport that responds to cyclic AMP. We wanted to determine whether aggregates of intramembranous particles appear in the cultured cells.

If aggregates appeared, the culture technique could be used as a source of material for subsequent biochemical approach. If, on the contrary, cultured cells lose their water sensitivity to ADH, it would be of interest to identify the defective step.

(2) Until now, only a few observations of the surface composition of amphibian urinary bladder have been made. No correlation between extrinsic and intrinsic proteins have been attempted. Dr. Pinto da Silva, Head of the Membrane Biology Section of the Laboratory of Pathophysiology, NCI, NIH, recently developed a new technique of fracture labeling which we will attempt to use for labeling intramembranous particle aggregates.

Results

We searched in $LLC\text{-}PK_3$ cells for the appearance of intramembrane particle aggregates after ADH challenge. We found that neither arginine vasopressin nor cyclic AMP and its active analogues modified the ultrastructure of this cell line. Some recent observations suggest that aggregates of intramembranous particles exist in cytosol vacuoles in unstimulated cells and are inserted into the apical membrane following ADH. Vesicles of preformed aggregates were not found in the cultured cells. These negative results are comparable to the observations previously made on cultured toad bladder cells which also are unaffected by vasopressin.

Proposed Course

(1) The ability of the fracture-labeling technique to visualize both the inner core of the membrane and the cytochemical markers should be very useful in defining the spatial arrangement of the membrane glycoproteins. Fracture labeling of toad bladder epithelial cells is currently in progress.

(2) Study of the structure and dynamics of intercellular contacts: Epithelial cells are linked together, at their apical pole, by well-developed intercellular contacts, the tight junctions. These intercellular contacts are not static structures since some physiological or pharmacological treatments can induce strong modifications in the pattern of the functional elements. We intend to study in further the structure and the dynamics of these intercellular contacts between cultured epithelial cells, under a variety of physiological as well as nonphysiological conditions, including the observations of cells treated with agents that alter the cytoskeleton.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01253-01 KE

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

The sodium transport response to aldosterone-regulation by thyroid hormone.

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

P.I.:	Raymond Pratt, M.D.	Div. Nephrology	AIR	WRH
Others:	J.S. Handler, M.D.	Section Chief	LKEM	NHLBI
	John P. Johnson, M.D.	Div. Nephrology	AIR	WRH

COOPERATING UNITS (if any)

Division of Nephrology, Walter Reed Army Institute for Research

LAB/BRANCH
Laboratory of Kidney and Electrolyte Metabolism

SECTION
Membrane Metabolism

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, Bethesda, Md.

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)

When epithelia formed in culture by cells by the continous line TB-6C are incubated with thryoid hormone (T_3) basal sodium transport rate (short-circuit current) is not altered although the sodium transport response to aldosterone is completely blocked. The effect of T_3 on receptors for aldosterone is under study.

PHS-6040
(Rev. 2-81)

Objectives

Previous work in this laboratory has shown that receptors for aldosterone can be studied with relative ease in aldosterone responsive cultured epithelia. It is the purpose of this study to characterize aldosterone receptors in epithelia formed by each of two lines of cells, TB-6c and TB-M, both derived from toad urinary bladder epithelial cells. In addition, since the sodium transport response of the toad bladder to aldosterone is inhibited by thyroid hormone (T_3) by an unknown mechanism, we will examine the effect of T_3 on the cultured epithelia.

Methods

See last years report (Z01 HL 01237-02 KE) for methods of measuring sodium transport rate (short-circuit current) and for methods of measuring nuclear bound aldosterone, presumed to reflect aldosterone bound to specific receptors that have migrated to the nucleus. Cytosolic receptors for aldosterone are assayed in a 15,000 xg supernatant solution prepared from a dounce homogenate using an isotonic homogenizing solution containing tungstate to prevent loss of receptor activity. The assay for cytosolic receptors is a minor modification of published methods using charcoal and dextran to precipitate unbound steroid at the end of the incubation with the cytosol fraction.

Major Findings

Incubation with 5 x 10^{-8}M T_3 for 48 hours has no effect on basal short-circuit current or resistance of TB-6c epithelia. The response to 10^{-7}M aldosterone(this concentration ordinarily yields maximal stimulation), however, is completely blocked. In epithelia of line TB-M, incubation with T_3 has no effect on basal short-circuit current but in contrast to line TB-6C, causes a marked fall in resistance, and has no effect on the response to aldosterone.

To assess the mechanism of the inhibition of the response to aldosterone, receptors for aldosterone are characterized. Initial experiments have established that the hypotonic lysis-filtration assay for nuclear bound aldosterone is suitable for epithelia formed by TB-6C cells. Specific nuclear binding of aldosterone is the same as that measured by more cumbersome centrifugation techniques that are convential.

Significance

This study should reveal whether thyroid hormone inhibits the sodium transport response to aldosterone by altering the number of cytosolic receptors for aldosterone(the initial site of interaction of aldosterone with the cell), the activation and migration of receptor bound aldosterone to the nucleus (the next step in the

action of aldosterone) or a later step. The absence of the inhibitory effect in line TB-M may enable us to distinguish the mechanism by which thyroid hormone acts in these epithelia.

Proposed Course

The binding sites for aldosterone in epithelia of TB-T6 will be characterized by affinity, number of sites, and affinity for steroid hormone analogs. These results will be correlated with data we have from earlier studies to determine which of the receptors is involved in the stimulation of sodium transport by aldosterone. Cytosolic and nuclear receptors will be assayed in epithelia incubated with thyroid hormone to see if thyroid hormone affects receptors for aldosterone.

Publications

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01254-01 KE

PERIOD COVERED

January 1, 1981 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Prolactin control of ion transport and metabolism in cultured mammary epithelia

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

P.I.:	C.A. Bisbee, Ph.D.	Guest Worker	LKEM	NHLBI
Other:	J.S. Handler, M.D.	Chief, MM	LKEM	NHLBI
	I.A. Mather, Ph.D.	Assoc. Prof.	DDS	UOM
	T. Oka		LBM	NIAMDD

COOPERATING UNITS (if any)

Department of Dairy Science, University of Maryland, College Park, Laboratory of Biochemistry and Metabolism, NIAMDD

LAB/BRANCH

Laboraory of Kidney and Electrolyte Metabolism

SECTION

Membrane Metabolism

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.9	0.9	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)

Mouse mammary epithelium is cultured on floating collagen gels. The lactogenic hormone prolactin causes increased sodium absorption. The effects will be correlated with the induction of milk-specific mRNA and products.

PHS-6040
(Rev. 2-81)

Objectives

The objective of these studies is to characterize the effects of prolactin on ion transport by mammary epithelium and to correlate these effects with the biochemical responses of this epithelium to prolactin. Although, the effects of prolactin on ion transport in vertebrates have been implicated in the mechanism of action of this hormone, it has been difficult to study electrolyte metabolism in mammary epithelium until the recent development of culture methods for maintaining differentiation *in vitro*. In these studies the diverse actions of prolactin on the mammary epithelium will be studied concurrently, thereby leading to a more complete understanding of the mechanism of action of prolactin. Additionally, the studies should lead to a better understanding of the hormonally controlled production of both the ionic and protein components of the physiologically important fluid milk.

Methods

Mammary epithelial cells will be maintained on collagen substrates that have been released and allowed to float in the medium. Cells cultured under these conditions assume an *in vivo* like cuboidal shape and show differentiated structure and function. Epithelium cultured in this manner will be studied using conventional techniques for electrophysiological measurements. Established techniques for the assay of milk-specific proteins and mRNA will be used (Dr. Oka). The hormonal induction of proteins will be studied using two-dimensional electrophoresis.

Measurement of apical membrane sodium channels will be done by quantifying displaceable (specific) and non-displaceable amiloride binding using equilibrium dialysis. These experiments will be performed with crude milk fat globule membrane and purified proteins derived from this membrane. The milk fat globule membranes are believed to be formed from the apical membrane of mammary epithelial cells. Mammary membranes that appear in milk have been studied extensively by Dr. Mather. He is making crude and purified preparations available for this study. Antibodies produced to the crude membrane fraction will be tested for their effects on binding of amiloride to membrane fractions and on mammary electrolyte transport using the short-circuit current technique.

Proposed Course

The effects of prolactin on cultured mammary epithelial cell electrophysiology will be further quantified and related to prolactin-induced production of milk-specific proteins and mRNA. Of particular interest is the time-course of prolactin induction of responses and the temporal relationship between the responses. In other studies, the specific binding of radiolabelled amiloride to milk proteins will be assessed. In addition available antibodies to specific milk proteins will be tested for an inhibitory effect on

transport by cultured mammary epithelium as an initial step in an attempt to isolate the amiloride sensitive sodium channel in the apical membrane of the epithelium.

Publications

None

Annual Report of the Laboratory of Molecular Cardiology
National Heart, Lung, and Blood Institute
October 1, 1980 to September 30, 1981

The Laboratory of Molecular Cardiology is investigating the regulation of contractile proteins in smooth muscle and non-muscle cells. We have also investigated the structure and enzymatic activity of myosin isolated from human hearts.

Regulation of myosin MgATPase activity by phosphorylation: (J. Sellers)

The various proteins involved in the reversible phosphorylation of smooth muscle and platelet myosin were purified to apparent homogeniety. These included: smooth muscle myosin kinase, smooth muscle phosphatase I, calmodulin, actin (from skeletal and smooth muscle) and myosin. Two sources of myosin were used, turkey gizzards and human platelets. In addition, the soluble myosin fragment, heavy meromyosin was prepared using smooth muscle myosin which was digested with chymotrypsin.

Starting with myosin in the unphosphorylated state the same myosin was phosphorylated, dephosphorylated and rephosphorylated, by addition and inhibition of the appropriate enzymes. In all cases there was a positive correlation between phosphorylation and the actin-activated MgATPase activity. This was true whether smooth muscle myosin or heavy meromyosin was used in the reconstituted system. The same positive correlation was also found for human platelet myosin.

When heavy meromyosin that was only partially phosphorylated was studied, a linear relationship between the extent of myosin phosphorylation and the actin-activated MgATPase activity was not found. Instead the curve obtained relating these two parameters suggested that the two heads of each myosin molecule had to be phosphorylated before the molecule could be activated by actin. The phosphorylation of these heads appears to be random.

Myosin kinase: (M.A. Conti)

Studies on the substrate-specific enzyme, myosin kinase, clarified the number of sites phosphorylated by cAMP-dependent protein kinase. When calmodulin was bound to myosin kinase, only one phosphorylated peptide could be isolated following extensive digestion of the enzyme with chymotrypsin. On the other hand, when myosin kinase was phosphorylated in the absence of bound calmodulin two peptides could be identified following two-dimensional peptide mapping. One of these peptides co-maps with the peptide phosphorylated when calmodulin was bound.

The same two ^{32}P-labelled peptides that were identified following digestion of the smooth muscle enzyme were also found when peptide mapping was carried out with a chymotyptic digest of phosphorylated human platelet myosin kinase. This work on the primary structure confirmed the previous work on the stoichiometry of ^{32}P-incorporation which had suggested that two different sites in myosin kinase could be phosphorylated by cAMP-dependent protein kinase.

Antibodies to myosin kinase: (P. de Lanerolle)

Antibodies to smooth muscle myosin kinase were purified on an affinity column of myosin kinase bound to Sepharose. These antibodies, which were raised in rabbits in response to avian myosin kinase, were found to inhibit the activity of human platelet myosin kinase. They were also found to localize to the stress fibers of a number of different non-muscle cells, including gerbil fibroma cells and human fibroblasts. This work was carried out by Primal de Lanerolle in collaboration with Jim Feramisco and Keith Burridge of Cold Spring Harbor. The antibodies are presently being used for injections into live cells in order to see if they will interfere with a defined cellular function (de Lanerolle in collaboration with Feramisco and Burridge, (gerbil fibrona cells) CSH and Keihart and Pollard, Johns Hopkins (mouse macrophages).

Phosphatases: (M. D. Pato)

Three different phosphatases have been purified from turkey gizzard smooth muscle. One of these was recently identified (phosphatase III) and appears to be relatively specific for smooth muscle myosin. Phosphatase I is a trimer (M_γ=60,000; 55,000; 38,000). The catalytic subunit (M_γ=38,000) has been dissociated from the other two subunits and purified by gel filtration. The catalytic subunit is active in dephosphorylating intact myosin and was used to dephosphylate myosin in the reconstitution experiments outlined above. In contrast the undissociated phosphatase does not dephosphorylate myosin.

Smooth muscle phosphatase I (undissociated) was used to dephosphorylate myosin kinase. Previously we had shown that phosphorylation of myosin kinase by protein kinase decreases the activity of this enzyme. Smooth muscle phosphatase I was used to dephosphorylate turkey gizzard and human platelet myosin kinase. In both cases it restored the myosin kinase activity found for the original unphosphorylated enzyme.

Structure and enzymatic studies of human cardiac myosin (J. Schier)

The presence of a fetal cardiac myosin light chain isoenzyme was confirmed. Myosin from infants that contained this light chain had one-half the actin-activated MgATPase activity of adult myosin as well as myosin purified from patients with hypertrophic obstructive cardiomyopathy (HOCM).

No evidence for a myosin heavy chain isoenzyme was found when infant, adult and HOCM peptide maps, generated by a number of different techniques, were compared.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01665-06 MC

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Smooth Muscle and Human Platelet Myosin Light Chain Kinase

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

PI:	Mary Ann Conti	Chemist	MC	NHLBI
	Robert S. Adelstein	Chief, Laboratory of Molecular Cardiology	MC	NHLBI
Other:	C. Robert Eaton	Physicist	MC	NHLBI
	William Anderson, Jr.	Chemist	MC	NHLBI
	J. Maurice Miles	Biological Laboratory Technician	MC	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Cardiology

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.4	1.4	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)

Smooth muscle myosin light chain kinase incorporates two moles of phosphate per mole of myosin kinase when it is incubated with the catalytic subunit of cAMP-dependent protein kinase and ATP in the absence of bound calmodulin. In the presence of bound calmodulin one mole of phosphate is incorporated per mole of kinase. Two dimensional peptide mapping on thin layer cellulose has confirmed the presence of two different sites of phosphorylation, only one of which can be phosphorylated when calmodulin is bound to myosin kinase. These two sites appear to be present in both turkey gizzard myosin kinase and human platelet myosin kinase. Using brief tryptic disgestion of native myosin kinase it was possible to first remove the two phosphorylated sites without loss of calmodulin dependence, and then the calmodulin binding site. The resulting fragment of myosin kinase was active in the absence of Ca^{2+}-calmodulin.

PHS-6040
(Rev. 2-81)

Project description: Myosin light chain kinase from turkey gizzard smooth muscle and human platelets is a substrate for the catalytic subunit of protein kinase. In the case of the smooth muscle enzyme, the site phosphorylated by protein kinase depends on whether calmodulin, which binds to myosin kinase in the presence of Ca^{2+}, is bound prior to phosphorylation. When calmodulin is bound, phosphate is incorporated into only 1 site on myosin kinase and the activity of the enzyme is not altered. When calmodulin is not bound during phosphorylation of myosin kinase, phosphate is incorporated into two sites and the affinity of myosin kinase for calmodulin is reduced 15-20 fold. The presence of two sites that can be phosphorylated in myosin kinase was confirmed by chymotryptic digestion and peptide mapping of the ^{32}P-labeled peptides.

Platelet myosin kinase has also been shown to undergo a decrease in activity following phosphorylation by protein kinase. This effect is reversible by dephosphorylation with a purified phosphatase. Similar to the smooth muscle enzyme, phosphorylation results in the incorporation of phosphate into two different sites. The peptides produced by extensive proteolysis of the ^{32}P labeled denatured myosin kinase co-map with those found following digestion of the smooth muscle enzyme.

When native smooth muscle myosin kinase is digested at 0°C with small amounts of trypsin, a 21,000 dalton fragment containing both phosphorylated sites is liberated. The remaining enzyme is still dependent on calcium and calmodulin for activity. Further digestion with trypsin converts this enzyme from a calcium-calmodulin-dependent kinase to one that is entirely independent of calcium-calmodulin. Present studies are directed at further characterizing the relationship between the active site of myosin kinase, the two sites of phosphorylation and the calmodulin-binding site.

Publications:

Adelstein, R.S. and Eisenberg, E.: Regulation and kinetics of the actin-myosin-ATP interaction. Ann. Rev. Biochem. 49: 921-956, 1980.

Scordilis, S.P., Uhlendorf, B.W., Scarpa, S., Cantoni, G.L., Miles, J.M. and Adelstein, R.S.: Changes in myosin and myosin light chain kinase during myogenesis. Biochemistry 20: 3511-3516, 1981.

Conti, M.A., and Adelstein, R.S.: The relationship between calmodulin binding and the phosphorylation of smooth muscle myosin kinase by the catalytic subunit of 3':5' cAMP-dependent protein kinase. J. Biol. Chem. 256: 3178-3182, 1981.

Hathaway, D.R., Eaton, C.R. and Adelstein R.S.: Regulation of human platelet myosin light chain kinase by the catalytic subunit of cyclic AMP-dependent protein kinase. Nature 291: 252-254, 1981.

Adelstein, R.S. and Klee C.B.: Purification and characterization of smooth muscle myosin light chain kinase. J. Biol. Chem. 256: (in press, 1981).

Hathaway, D.R., Adelstein, R.S. and Klee, C.B.: Interaction of calmodulin with myosin light chain kinase and cAMP-dependent kinase in bovine brain. J. Biol. Chem. (in press, 1981).

Bahn, A., Malhotra, A., Scheur, J., Conti, M.A., and Adelstein, R.S.: Subunit function in cardiac myosin: Effects of binding phosphorylated and unphosphorylated myosin light chain -2 to light chain -2 deficient myosin. J. Biol. Chem. (in press, 1981).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01752-03 MC

PERIOD COVERED: October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Purification of Myosin Light Chain Phosphatases

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

PI:	Mary D. Pato	Visiting Fellow	MC	NHLBI
Other:	R. S. Adelstein	Chief, Laboratory of Molecular Cardiology	MC	NHLBI
	C. R. Eaton	Physicist	MC	NHLBI
	W. Anderson, Jr.	Chemist	MC	NHLBI
	J. M. Miles	Biological Laboratory Technician	MC	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Cardiology

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 2.2	PROFESSIONAL: 1.2	OTHER: 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)

Three different phosphatases have been purified from smooth muscle. Phosphatases I and II dephosphorylates myosin light chain and myosin kinase but not intact myosin. Phosphatase III dephosphorylates both myosin light chain and intact myosin at comparable rates which are higher than that for myosin kinase. The catalytic subunit of phosphatase I dephosphorylates intact myosin.

PHS-6040
(Rev. 2-81)

Project Description: Myosins isolated from smooth muscle and non-muscle cells require phosphorylation of their 20,000 dalton light chain in order to undergo actin-activation of their MgATPase activity. The enzyme that catalyzes the phosphorylation, myosin kinase, is a substrate of cAMP-dependent protein kinase. Incorporation of two moles of phosphate into the myosin kinase decreases its activity.

The phosphatases that dephosphorylate smooth muscle myosin and myosin kinase were purified from smooth muscle turkey gizzards and characterized. Smooth muscle phosphatase I (SMP-I) is a trimer of polypeptides with molecular weights of 60,000, 55,000 and 38,000. SMP-I dephosphorylates myosin light chain and myosin kinase but not intact myosin. However, the dissociated catalytic subunit of SMP-I, which was identified as the 38,000 dalton subunit, dephosphorylates the intact myosin. SMP-II is a 43,000 polypeptide enzyme which requires Mg^{2+} for activity. It preferentially dephosphorylates myosin light chain compared to myosin kinase and has no activity towards intact myosin. SMP-III dephosphorylates intact myosin and myosin light chain at comparable rates which is higher than that for dephosphorylation of myosin kinase.

The catalytic subunit of SMP-I was used as one of the enzymes for the reconstitution of the myosin phosphorylating system to study the correlation of reversible phosphorylation of myosin from smooth muscle and non-muscle cells with their actin-activated MgATPase activity. Fully phosphorylated myosin was maximally activated by actin while unphosphorylated and dephosphorylated myosin cannot be actin-activated. SMP-I was also used to reverse the decreased activity of myosin kinase isolated from smooth muscle and platelets, caused by phosphorylation with cAMP-dependent protein kinase.

We are currently studying the regulation of the smooth muscle phosphatases.

Publications: Adelstein, R.S., Conti, M.A., and Pato, M.D.: Regulation of myosin light chain kinase by reversible phosphorylation and calcium-calmodulin. Annals N.Y. Acad. Sci. 356: 142-150, 1980.

Adelstein, R.S., Pato, M.D., and Conti, M.A.: The role of phosphorylation in regulating contractile proteins. Adv. in Cyclic Nucleotide Research 14: 361-373, 1981.

Adelstein, R.S., Pato, M.D., Sellers, J.R., Conti, M.A., and Eaton, C.R.: Regulation of contraction by reversible phosphorylation of myosin and myosin kinase. Cold Spring Conferences on Cell Proliferation, Vol. 8 (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01785-02 MC

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Studies with Antibodies to Myosin Kinase Isolated from Smooth Muscle

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

PI:	Primal de Lanerolle	Staff Fellow	MC	NHLBI
Other:	Robert S. Adelstein	Chief, Laboratory of Molecular Cardiology	MC	NHLBI
	William Anderson	Chemist	MC	NHLBI
	J. Maurice Miles	Biological Laboratory Technician	MN	NHLBI

COOPERATING UNITS (if any)
Drs. James Feramisco and Keith Burridge, Cold Spring Harbor Labs., Cold Spring Harbor, N.Y.
Drs. Dan Kiehart and Thomas Pollard, Johns Hopkins Medical School., Baltimore,/Md.

LAB/BRANCH
Laboratory of Molecular Cardiology

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.7	PROFESSIONAL: 1.2	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)

Antibodies to the enzyme myosin light chain kinase have been purified, characterized and used to study the role of this enzyme in regulating smooth muscle contraction as well as contractile activity in non-muscle cells. Immunofluorescence studies have demonstrated that myosin kinase is localized on stress fibers of cultured cells. Data from experiments in which living cells were microinjected with these antibodies suggest that myosin kinase plays an important role in regulating contractile activity in non-muscle cells.

PHS-6040
(Rev. 2-81)

Project Description: We have been studying the role of myosin phosphorylation in regulating contraction in smooth muscle and contractile events in non-muscle cells.

The probe used in these studies has been a population of heteroclonal antibodies produced by inoculating both rabbits and goats with myosin kinase purified from turkey gizzard smooth muscle. These antibodies have been purified and extensively characterized. (see publication). Immunodiffusion experiments indicate that these antibodies cross-react with purified turkey gizzard myosin kinase. Other experiments have demonstrated that these antibodies immunoprecipitate purified myosin kinase or myosin kinase found in tissue extracts prepared from avian or mammalian smooth muscles. Biochemical experiments have shown that these antibodies inhibit the catalytic activity of smooth muscle and non-muscle myosin kinases. We, in collaboration with Drs. James Feramisco and Keith Burridge, have also used these antibodies to demonstrate that myosin kinase has the same intracellular distribution as myosin in cultured cells.

These antibodies have also been used to study the physiological role of myosin in regulating contractile events in living tissues. We have microinjected antibodies to myosin kinase into macrophages (studies performed in collaboration with Drs. Dan Kiehart and Thomas Pollard). Data from initial experiments indicate that these antibodies, presumably through the inhibition of myosin kinase activity, may have a profound effect on cell motility and shape. Other microinjection experiments, performed in collaboration with Dr. James Feramisco, suggest that myosin kinase may be important in maintaining the integrity of intracellular filaments in cultured gerbil fibroma cells.

Finally, experiments are in progress to determine whether myosin dephosphorylation and myosin kinase phosphorylation are correlated with relaxation of tracheal smooth muscle. Antibodies to myosin and myosin kinase will be used to immunoprecipitate these proteins from pieces of muscle frozen during relaxation. The phosphate content of these proteins will then be quantitated in order to determine the role of myosin dephosphorylation and myosin kinase phosphorylation in receptor mediated relaxation of tracheal smooth muscles.

Publications: de Lanerolle, P., Adelstein, R.S., Feramisco, J.R., and Burridge, K.: Characterization of antibodies to smooth muscle myosin kinase and their use in localizing myosin kinase in non-muscle cells. Proc. Natl. Acad. Sci. (USA) (In press, 1981).

Feramisco, J.R., Burridge, K., de Lanerolle, P., and Adelstein, R.S.: Localization of myosin light chain kinase in non-muscle cells. Cold Spring Harbor Conference on Cell Proliferation. Vol. 8, (In press, 1981).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01786-02 MC

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Role of Phosphorylation as a Regulatory Mechanism in Muscle Contraction

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

PI:	James R. Sellers, Jr.	Staff Fellow	MC	NHLBI
Other:	Robert S. Adelstein	Chief, Laboratory of Molecular Cardiology	MC	NHLBI
	William Anderson	Chemist	MC	NHLBI
	J. Maurice Miles	Biological Laboratory Technician	MC	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Cardiology

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.7	1.2	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 mechanism of phosphorylation-dependent myosin-linked regulation is being investigated using myosin and its subfragments from turkey gizzard. This problem is being attacked using a variety of steady state and transient kinetic techniques.

PHS-6040
(Rev. 2-81)

Project Description: In order to address the question as to whether smooth muscle myosin can be reversibly regulated by a calcium-dependent phosphorylation of the regulatory light chains, we performed an experiment using only purified, well characterized proteins. Turkey gizzard myosin was isolated in an unphosphorylated state. It was phosphorylated, dephosphorylated, and rephosphorylated using purified myosin kinase and phosphatase. The actin-activated MgATPase activity of the unphosphorylated and dephosphorylated myosin was low whereas that of the phosphorylated and rephosphorylated was high. Neither the MgATPase in the absence of actin nor the high salt K+EDTA ATPase activity were altered by phosphorylation. This indicates that phosphorylation alone is capable of regulating the actin-activated MgATPase of smooth muscle myosin. Similar experiments performed using a non-muscle myosin from human platelets indicated that this myosin is also reversibly regulated by phosphorylation.

Myosin is insoluble at low ionic strengths and is thus not amenable for many types of enzymatic analysis. Therefore we prepared the soluble two-headed subfragment, heavy meromyosin (HMM). It was found that chymotryptic digestion of phosphorylated gizzard myosin resulted in an HMM preparation which retain virtually all of its regulating light chain in a phosphorylatable form. This was a significant advance over the published preparations for smooth muscle HMM. We then established the fact that this HMM was reversibly regulated by a phosphorylation/dephosphorylation of its regulatory light chains. This indicates that filament formation is not essential for regulation in smooth muscle myosin (HMM). The HMM preparation will be used to examine the mechanism of the phosphorylation-dependent regulation using equilibrium binding studies and transient kinetic techniques in collaboration with Drs. Evan Eisenberg and Lois Greene.

Recently experiments have been performed to determine the mechanism of activation by phosphorylation with myosin and HMM. It was found that for both myosin and HMM both heads of the enzyme must be phosphorylated before the MgATPase of either head could be activated by actin.

Publications: Sellers, J.R.: Phosphorylation dependent regulation of *Limulus* myosin. J. Biol. Chem. (in press, 1981).

ANNUAL REPORT OF THE
MOLECULAR DISEASE BRANCH
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
OCTOBER 1, 1980 THROUGH SEPTEMBER 30, 1981

The overall objective of the research program of the Molecular Disease Branch is the delineation of the molecular and structural properties of the human plasma apolipoproteins, the physiological role of the apolipoproteins and lipoproteins in lipid transport, the determination of the mechanisms involved in the regulation of cellular cholesterol metabolism and transport, and the elucidation of the metabolic and molecular mechanisms involved in plasma lipoprotein biosynthesis, transport, and catabolism in normal individuals and patients with disorders of lipid metabolism and atherosclerosis.

During the last several years the staff of the Molecular Disease Branch has developed a conceptual framework for the understanding of the dynamic processes involved in the biosynthesis, transport, and catabolism of plasma apolipoproteins. Within this framework the plasma lipoproteins are conceptualized as a polydisperse collection of lipoprotein particles, the lipoprotein composition of which is determined by the laws of mass action. The important molecular constituent of the plasma lipoprotein particle which is involved in the regulation of lipoprotein particle transport and metabolism is the apolipoprotein moiety. The distribution of a specific apolipoprotein within plasma is governed by the relative concentrations of and affinity for, the plasma lipoproteins. This concept of plasma lipoproteins emphasizes the fundamental importance of the apolipoprotein in regulating lipoprotein metabolism and provides a model for understanding apolipoprotein-lipoprotein interactions during lipoprotein biosynthesis, transport, and catabolism in normal subjects and in patients with dyslipoproteinemia and atherosclerosis.

Prerequisite to an analysis of the physiological role of plasma apolipoproteins in lipid metabolism is a detailed knowledge of the biochemistry and molecular structure of the plasma apolipoproteins. Over the last several years we have undertaken a systematic study of the human plasma apolipoproteins including the primary structure of apoA-I, apoA-II, apoC-I, and apoC-III. Recently these studies have been extended to an analysis of apoA-$I_{Tangier}$ and apoA-$II_{Tangier}$ isolated from a patient homozygous for Tangier disease. ApoA-$I_{Tangier}$ differed from apoA-I isolated from normal subjects in amino acid composition, apparent molecular weight on SDS gel electrophoresis, and heterogeneity of isoforms on two-dimensional polyacrylamide gel electrophoresis. ApoA-$II_{Tangier}$ was identical in physico-chemical properties to apoA-II from normal subjects. These results are consistent with the concept that the molecular defect in Tangier disease is due to a structurally abnormal A-I apolipoprotein.

During the last year research has continued to focus on apoE. ApoE binds to a high affinity liver receptor and has been proposed to be responsible for the hepatic clearance of chylomicron remnants. It is now recognized that there are three alleles for apoE. The three alleles code for apoE_2, apoE_3, and apoE_4. Individuals homozygous for apoE_2 (apo$E_{2,2}$) have a Type III phenotype.

ApoE has been isolated in homogeneous form from patients homozygous for apoE_2, apoE_3, and apoE_4. The individual apoE isoforms differ by a single charge and are readily separated by isoelectric focusing. When compared to apoE_4, apoE_2 was shown to have delayed catabolism in normal and Type III subjects and

in rat hepatocyte cells in culture. Based on these results we have proposed that the molecular defect in Type III hyperlipoproteinemia is due to a structural defect in apoE which results in delayed catabolism of chylomicron remnants (see below).

Of particular interest over the last year has been the critical analysis of the B apolipoprotein. Two B apolipoproteins, designated apoB-I and apoB-II, can be separated by SDS gel electrophoresis. ApoB-I or large apoB, is the predominate apolipoprotein in LDL and is thought to be synthesized in the liver. ApoB-II, or small apoB, is the major B apolipoprotein in thoracic duct chylomicrons, and has been proposed to be synthesized primarily by the intestine. ApoB-I and apoB-II have been purified by preparative SDS gel electrophoresis. The amino acid composition of apoB-I and apoB-II showed no cross reactivity. These results are interpreted as indicating that apoB-I and apoB-II may represent two separate distinct apolipoproteins. ApoB-I may represent the major structural proteins associated with particles synthesized by the liver, whereas apoB-II is the principal structural protein of lymph chylomicrons synthesized by the intestine.

ApoH is a newly recognized plasma apolipoprotein, which is associated with triglyceride-rich lipoproteins in thoracic duct lymph and plasma. ApoH has been shown to bind to artificial lipid emulsion and to have a high affinity for plasma lipoproteins. Detailed characterization of apoH indicated that it was identical to β_2-glycoprotein-I which has been described several years ago as a glycoprotein component of plasma. Of particular importance was our discovery that apoH activated lipoprotein lipase. Thus, both apolipoproteins H as well as C-II modulate the enzymic activity of lipoprotein lipase and triglyceride metabolism.

During the last year significant progress has been made in the methods for the solid phase synthesis of proteins. The use of the phenylacetoamidomethyl (PAM) resin has improved acid stability, and the insertion of the PAM group between the peptide and the polystyrene matrix reduces the rate of peptide loss from the resin to only 1% of that of the conventional styrene-divinylbenzene resin, and enhances the applicability of the PAM resin for the synthesis of longer proteins. The PAM resin was employed in the solid phase synthesis of the intact 84 amino acid polypeptide hormone, human parathyroid hormone (h-PTH). The synthesized hormone had complete biological activity and showed complete immunological cross reactivity with native hPTH. The ability to synthesize proteins of 80 to 100 amino acids in length will markedly facilitate our ability to ascertain the amino acid residues required for lipid binding, protein-receptor interaction, and enzyme-cofactor function of the plasma apolipoproteins.

A systematic analysis of the molecular properties of the human apolipoproteins continues to be an active area of research within the Branch. A detailed knowledge of the physico-chemical properties of the apolipoproteins is required for our understanding of the molecular mechanisms involved in apolipoprotein-lipoprotein particle interaction and metabolism. Within the framework of our concept of lipoproteins the quaternary structure of plasma lipoproteins and the changes in apolipoprotein composition of plasma lipoproteins during transport and metabolism is dependent on the molecular properties of the individual apolipoproteins. Studies within our Branch over the last several years have revealed that the human apolipoproteins have several unique molecular properties. Of particular importance was the finding that apolipoproteins undergo self- as well as mixed association. Detailed studies in the Branch have establilshed the following self-association schemes for the individual apolipoproteins: apoA-I, monomer-dimer-

tetramer-octamer; apoA-II, monomer-dimer; reduced and carboxymethylated apoA-II, monomer-dimer; apoC-I, monomer-dimer-tetramer; and apoC-III, monomer-dimer-trimer. Mixed associations between individual apolipoproteins include: apoA-I:apoA-II 1:1, 2:2; apoA-II: apoC-I, 2:4; and apoA-I:apoC-I, 2:4.

Of particular note was the recognition that concomitant with self-association of the apolipoproteins was a major increase in molecular structure. In the monomeric form, apolipoproteins contain little organized structure, whereas in the oligomeric form the conformation increased to that characteristic of globular proteins. These dramatic changes in conformation are greater than those reported for any other self-associating protein system. The driving force for the dramatic change in conformation of the apolipoproteins is the shielding of non-polar residues from the solvent and is thus hydrophobic in nature.

The self-association of plasma apolipoproteins has been shown to be exquisitely sensitive to various ligands, pH, ionic strength, and protein concentration. Of pariticular note has been the demonstration of hydrostatic pressure on the molecular properties of the apolipoproteins. In interacting systems, when there is a major change in solvent exposure of the non-polar residues during oligomer formation, there may be a significant change in molar volume. This is primarily due to the unfavorable interaction between non-polar solutes and water. Presumably decreases in the amount of "order water" allow non-polar solutes to expand when shielded from solvent. The self-association of apoA-I, apoA-II as well as apoC-I have been shown to be significantly effected by pressure and the volume changes on associations are of a magnitude greater than previously reported in the literature.

The mixed association of apolipoproteins may play an important role in the physiological function of apolipoproteins. Studies have been reported which show that the C apolipoproteins inhibit the hepatic clearance of chylomicron remnants. This may represent a specific protein-protein mixed association which modulates the uptake of lipoprotein particles by the liver.

During the past year the analysis of the molecular properties of apolipoproteins has been extended to apoH. ApoH has a molecular weight of 43,400 in non-denaturing buffers and 42,600 in guanidinium chloride. The circular dichroic spectrum was unusual with no strong maximum near 200 nm or major transitions between 208-222 nm. The major features of the spectrum include a positive maximum at 235 nm and a weak maximum at 205 nm. At neutral pH the affinity of apoH for an air-water interphase is comparable to gobular proteins. Acidification or alkalinization of solutions of apoH induced only minor changes in the circular dichroic spectra, however a major increase in affinity for the air-water interface occurs such that apoH resembles the more flexible apolipoproteins (e.g., apoA-I and apoA-II). ApoH exhibited no self-association under any conditions tested, thus distinguishing it from the other apolipoproteins that have been investigated. The unique physico-chemical properties of apoH may be related to the unusually high content of cystine and proline in the protein. The importance of the unique physico-chemical properties of apoH to its ability to function as a cofactor for lipoprotein lipase remains to be ascertained.

The elucidation of the molecular properties of the apolipoproteins will be fundamental to our understanding of the structure and function of the apolipoprotein as well as the molecular organization of the plasma lipoprotein particles.

Our ultimate understanding of lipoprotein metabolism will necessiate a detailed knowledge of the quaternary structure of plasma lipoprotein particles.

During the last several years the interaction of plasma lipoproteins with cells grown in culture has attracted a great deal of interest as well as research. Cellular binding, uptake, and degradation of lipoprotein particles appears to be mediated by both apolipoproteins B-I and E. ApoB-I appears to be the major apolipoprotein involved in the uptake of lipoproteins in peripheral cells including fibroblasts, smooth muscle cells, and endothelial cells. The E apolipoprotein is important in the hepatic uptake of chylomicron remnants.

Lipoprotein interactions with cells can be divided into those that result in reversible as well as irreversible cell interactions. A new kinetic treatment for ligand-cell interactions was developed which permitted the analysis of data obtained from irreversible ligand-cell interactions. The new analysis permits in theory the calculation of the relative affinity and total number of receptors for systems exhibiting irreversible interactions. The intercept of a Scathard type of analysis for irreversible systems is the product of the number of binding sites and the rate of internalization. Application of this new kinetic analysis to apoB-I (LDL) and LpE (HDLc) binding to fibroblasts suggests that the binding of both lipoprotein particles exhibit positive cooperativity. These results are consistent with recent observations by electron microscopy on the clumping of colloid gold-complexed LDL.

A new method has also been developed during the last year for the direct quantitation of reversible lipoprotein particle cell-interaction. A new microculture technique for growing fibroblasts attached to beads has been developed. Using this method a new assay for reversible binding was developed by rapid filtration. This new assay method will permit the delineation of binding affinities and number of receptor sites involved in lipoprotein-cell interactions.

The intracellular events initiated by the cellular binding of lipoproteins has been extensively investigated over the last several years. Of increasing interest and importance is the intracellular regulation of cholesterol esterification, hydrolysis, and biosynthesis. Key enzymes involved in the regulation of the intracellular concentration of free cholesterol are acid and neutral ester hydrolase. Acid ester hydrolase (AEH) is the lysosomal enzyme responsible for hydrolysis of cholesterol esters into cholesterol and free fatty acids which are transported into the cell associated with LDL. Neutral ester hydrolase (NEH) is the cytosolic enzyme responsible for the hydrolysis of cholesterol esters formed by the action of acyl-cholesterol acyltransferase (ACAT). New sensitive methods were developed to quantitate AEH and NEH in nonhepatic tissue including fibroblasts growing in culture. These methods were applied to the analysis of cells from a patient with Wolman's disease. Wolman's disease is characterized by steatorrhea, hepatosplenomegaly, absence of AEH and death in infancy. Fibroblasts from a patient with Wolman's disease and control cells were grown in culture and assayed for both AEH and NEH. AEH activity was not detected, however NEH activity persisted consistent with the view that these two enzymes were independently coded and synthesized.

AEH has also been extensively evaluated in the mononuclear cells of patients (age 24-45) with angiographically documented coronary artery disease. AEH activity was significantly lower in the patients with premature cardiovascular disease as compared to controls. Two well-established risk factors, smoking and

low HDL-cholesterol were also statistically different between the control and premature cardiovascular group. However, multivariant analysis revealed the reduction in activity of AEH to be significant ($p<.005$) and independent of the incidence of HDL-cholesterol and smoking. These studies clearly establish the importance of a detailed analysis of the enzymes involved in intracellular cholesterol metabolism. A number of separate molecular defects in these enzyme systems may be responsible for diseases characterized by an increase in intracellular cholesterol (ester), foam cells, and premature cardiovascular disease.

One of the other major enzymes involved in intracellular cholesterol metabolism is HMG-CoA reductase, the rate limiting enzyme in the pathway for cholesterol biosynthesis. During the last several years the factors involved in the modulation of the enzymic activity of HMG-CoA reductase has been extensively studied in the Branch. HMG-CoA reductase has been isolated in electrophoretically homogeneous form from chicken and rat liver. HMG-CoA reductase was shown to be present in enzymically active and inactive forms. Detailed studies established that the reversible inactivation of HMG-CoA reductase was due to covalent modification of the enzyme by a reversible phosphorylation-dephosphorylation reaction sequence.

The enzyme, reductase kinase, which catalyzes the phosphorylation of HMG-CoA reductase has also been purified to homogeneity. Reductase kinase, like HMG-CoA reductase, was shown to undergo reversible activation-inactivation. Studies employing a partially purified cytosolic protein kinase designated reductase kinase kinase, phosphoprotein phosphatase, and [^{32}P]-ATP have established that reductase kinase undergoes reversible phosphorylation-dephosphorylation. These results provide evidence that the *in vitro* enzymic activity of both HMG-CoA reductase and reductase kinase are modulated by a phosphorylation-dephosphorylation reaction sequence. The active form of HMG-CoA reductase is dephosphorylated, wherease the phosphorylated form of reductase kinase is active.

The regulation of HMG-CoA reductase by reversible phosphorylation was established *in vitro*, and then extended to *in vivo* studies in the rat. *In vivo* phosphorylation of HMG-CoA reductase was established by purification of the enzyme following injection of [^{32}P] into rats. Glucagon administration was associated with a decrease in enzyme activity and increase in phosphorylation of HMG-CoA reductase. These studies were interpreted as indicating that HMG-CoA reductase undergoes phosphorylation *in vivo*, and the degree of phosphorylation of HMG-CoA reductase may be modulated by polypeptide hormones.

Two additional effectors have now been identified which modulate the short-term regulation of HMG-CoA reductase by reversible phosphorylation. Cholesterol and mevalonolactone were administered to rats and shown to acutely reduce the activity of HMG-CoA reductase by increasing the degree of phosphorylation of the enzyme. Therefore glucagon, cholesterol, and mevalonolactone have been shown to effect the activity of HMG-CoA reductase on a short-term basis by modulating the reversible phosphorylation of HMG-CoA reductase.

The modulation of the activity of HMG-CoA reductase has now been extended to an analysis of the regulation of the enzyme in human liver. HMG-CoA reductase was purified to homogeneity from human liver. Human HMG-CoA reductase was shown to undergo reversible activation-inactivation by reversible phosphorylation. In addition, reductase kinase was shown to be present in human liver and exist in active-inactive forms due to reversible phosphorylation. Evidence was also

obtained for the presence of reductase kinase kinase in human liver. These results represent the first demonstration of the *in vitro* modulation of human liver HMG-CoA reductase by a bicyclic cascade system involving reversible phosphorylation.

Regulation of HMG-CoA reductase by a bicyclic cascade system has now been established in the human as well as the rat. The overall regulation of the biosynthesis of cellular cholesterol involves short-term regulation by the bicyclic cascade system, and long-term regulation by changes in enzyme concentration due to changes in biosynthesis and/or degradation.

The synthesis, transport, and catabolism of plasma lipoproteins continues to be an active area of investigation within the Branch. Two major enzymes, lipoprotein lipase (LPL) and hepatic lipase (HL), present in post-heparin plasma (PHLA) are responsible for the lipolysis of triglycerides in VLDL and chylomicrons. Research has recently focused on the role of HL in lipid metabolism. Plasma was shown to activate HL. Systematic analysis of plasma and apolipoprotein components established that apoA-II was the *in vitro* activator of HL. ApoA-II is therefore a cofactor for the activity of HL in much the same manner as apoC-II is a cofactor for LPL. Our studies, like studies from other laboratories, have suggested that one of the major substrates for HL is HDL. The activity of HL appears to be inversely correlated with the plasma concentration of HDL. The activity of HL was also correlated with HDL in patients with primary biliary cirrhosis. Two groups of patients were identified. One group had decreased HL activity and increased levels of HDL, apoA-I and apoA-II. The second group had decreased HDL, and an increase in the lipoprotein, LpX, which is characteristic of advanced obstructive liver disease.

The elucidation of the role of HL in the metabolism of HDL and the recognition of apoA-II as the cofactor for HL provides new insights into the regulation of HDL metabolism. Based on this new information we have proposed a new comprehensive metabolic scheme for HDL metabolism. Major pathways for the production of HDL constituents include the metabolism of triglyceride-rich lipoproteins by LPL with apoC-II as a cofactor. The lipid and apolipoprotein constituents increase HDL_3, and are associated with the conversion of HDL_3 to HDL_2 particles. The enzyme LCAT, with apoA-I as a cofactor, increases the cholesterol ester content of HDL_2. HL, with apoA-II as a cofactor, catalyzes the hydrolysis of HDL_2 with ultimate uptake of lipid-apolipoprotein constituents into tissues (e.g., liver) and the conversion of HDL_2 to HDL_3. Phospholipid and cholesterol ester exchange proteins also transfer lipid constituents from HDL to LDL and triglyceride-rich lipoproteins. This combined new scheme for HDL metabolism involves a reversible conversion of HDL_2 and HDL_3 and three enzymes including HL, LPL, and LCAT, each of which is modulated by a specific cofactor (apoA-II, apoC-II, and apoA-I respectively).

The role of apolipoproteins in the metabolism of plasma lipoproteins continues to be a focal point of study since the apolipoprotein is the unique constituent of the lipoprotein particle. The physiological function of several apolipoproteins has now been established. Apolipoproteins are known to serve as cofactors for enzymes (apoC-II and lipoprotein lipase, apoA-I and lecithin cholesterol acyltransferase, as outlined above), ligands for receptor binding (apoB and apoE), and exchange proteins (apoD-cholesterol ester exchange). Of particular interest are recent studies on the newly recognized apolipoprotein constituent of triglyceride-rich lipoproteins, apoH (β_2-glycoprotein-I). ApoH

has been isolated in homogeneous form as outlined above, and shown to be a constituent of chylomicrons and VLDL. ApoH was shown to activate lipoprotein lipase (LPL), the major enzyme involved in the hydrolysis of triglycerides of chylomicrons and VLDL. The elucidation of the function of the individual apolipoproteins in lipoprotein metabolism will markedly facilitate our understanding of lipoprotein metabolism in normal subjects and patients with dyslipoproteinemia and atherosclerosis.

Quantification of apolipoprotiens represents a major prerequisite to the analysis of apolipoprotein concentration and metabolism in normal subjects and patients with dyslipoproteinemia. Immunochemical assays employing radial immunodiffusion, radial immunoassay or rocket electrophoresis have been developed and tested for several of the apolipoproteins including apoA-I, apoA-II, apoC-II, apoC-III, and apoB-I. New assays for apoH, apoE, and apoA-IV have been developed during the last year. Quantification of apolipoproteins in normal individuals and patients with disorders of lipid metabolism and atherosclerosis are presently in progress.

The synthesis, transport, and catabolism of plasma lipoproteins in normal subjects and patients with dyslipoproteinemia continues to be actively investigated within the Branch. Two new diseases have been discovered during the last year. The first new disease is a deficiency of the E apolipoprotein. The proband was a 60 year-old female with a 10-year history of tubo-eruptive xanthomas, 3-year history of angina pectoris, and documented coronary artery disease by angiography. The patient's plasma cholesterol and triglyceride were increased, and she was diagnosed as having Type III hyperlipidemia with a fast β band on agarose electrophoresis and a VLDL cholesterol to plasma triglyceride ratio of $>.3$. Detailed analysis by gel electrophoresis and immunochemical techniques of the patient's apolipoproteins in plasma and in the separated VLDL, LDL, and HDL density fractions revealed an absence of apoE. Three members of the kindred have been shown to have Type III hyperlipidemia and an absence of apoE. ApoE has been proposed to be important in the uptake of chylomicron remnants by the liver as outlined above. A deficiency of the E apolipoprotein results in a delayed clearance of chylomicron remnants and a Type III hyperlipoproteinemia. A deficiency of apoE therefore represents a new cause for Type III hyperlipoproteinemia.

The second new disease was discovered in a 45 year-old female with a 3-year history of corneal opacification and a 3-month history of angina pectoris. Coronary angiography documented severe coronary artery disease. The proband had a normal level of plasma triglycerides, a low level of plasma cholesterol, and a virtual absence of HDL. Detailed analysis of the patient's plasma revealed an absence of apoA-I, and low plasma levels of apoA-II and apoC-II. A deficiency of apoA-I was therefore associated with a severe reduction in HDL. Low concentrations of HDL have been previously reported to be associated with an increase in premature coronary artery disease. ApoA-I deficiency therefore represents a new cause for a severe reduction in HDL, ultimately leading to severe coronary artery disease.

During the last several years our Branch has extensively analyzed the metabolism of apoA-I and apoA-II. These studies have provided detailed information on the metabolism of these two apolipoproteins which represent the two major proteins within HDL. Several new concepts have developed during these studies which include the following: radiolabeling of A-I and A-II as apolipoproteins gave similar results as radiolabeling the intact HDL particle; this is of importance since it

established our proposal that apolipoproteins could be utilized for studying the metabolism of lipoproteins; the mean residence time of apoA-I in plasma and HDL is significantly shorter than apoA-II; the increased apoA-I levels present in female subjects are due to increased apoA-I biosynthesis; the plasma residence time of apoA-I is inversely correlated with plasma triglyceride levels and is an important determinant of plasma apoA-I concentration in both females and males.

A compartmental model for apoA-I and apoA-II was constructed for males and females using radiolabeled apolipoprotein kinetic data from plasma, urine, and whole body counter. ApoA-II decayed by two pathways in both males and females. In males 25% of apoA-II decayed through a minor pathway which was significantly longer (22 hrs) than the major pathway. In females 30% of apoA-II was metabolized through the minor slow pathway. ApoA-I had two distinct plasma compartments in both male and females. The major component had a residence time in males and females of 5.4 days and 6.8 days respectivley. The minor component contained 28% and 40% of the apoA-I in males and females respectively. The modeling studies clearly demonstrate the heterogeneity of the metabolism of apoA-I and apoA-II in both males and females. Detailed analysis are therefore critical to the ultimate understanding of the factors modulating the metabolism of apoA-I and apoA-II in normal subjects as well as subjects with dyslipoproteinemia.

Research on apoE continues to be a major focus of the Branch during the last year. $ApoE_2$, E_2, and E_4 have been isolated in homogeneous form and detailed clinical studies are continuing on the metabolism of the individual isoforms. Kinetic analysis has indicated that $apoE_2$ has a residence time twice as long as $apoE_4$ in both normal subjects as well as patients with Type III hyperlipoproteinemia. The elevated plasma levels of apoE in Type III patients were shown to be due entirely to a catabolic defect as the synthetic rates of apoE were normal. Rat hepatocytes were found to bind $apoE_4$ much more avidly than $apoE_2$. As noted above, these combined findings are consistent with our hypothesis that the hyperlipidemia in Type III hyperlipoproteinemia is due to an abnormal apoE allele (e.g., $apoE_2$) with a delayed metabolism of chylomicron remnants due to the abnormal E apolipoprotein.

Clinical studies are also continuing on triglyceride metabolism in a variety of different populations including diabetic and non-diabetic Pima Indians, familial hypertriglyderidemia, and combined hyperlipidemia. In monogenic familial hypertriglyceridemia the transport of triglycerides were not increased and the elevated triglycerides were secondary to a decreased fractional catabolic rate. In familial combined hyperlipidemia the elevated triglycerides were due either to an increased production rate or in a few patients, secondary to a decreased fractional catabolic rate.

An additional disease which has been studied in detail during the last year is beta-sitosterolemia. Patients with this disorder have xanthomas in childhood, premature cardiovascular disease, and leveled plasma plant sterols. Detailed balance studies revealed that these patients also overabsorb shellfish sterols. These results are consistent with the view that these patients have a generalized overabsorption of all dietary sterols. Studies on these patients were extended to the cellular level. Skin fibroblast were grown in culture, and analyzed for LDL binding and intracellular enzyme function. LDL binding and internalization were normal. AEH, NEH, and HMG-CoA reductase were all similar in activity to control cells. The molecular defect in this disease now appears to be due to an

abnormality at the site for discrimination for sterol absorption. In normal individuals plant and shellfish sterols are not absorbed whereas cholesterol is actively transported into the body. In patients with beta-sitosterolemia there is no selectivity to sterol absorption and cholesterol, plant, as well as shellfish sterols are absorbed in significant quantities.

One of the major aims of the staff of the Branch is the effective treatment of hyperlipidemia with the ultimate goal of reducing blood lipids and preventing premature cardiovascular disease. To this end we have initiated a new outpatient clinical trail for the treatment of patients with familial hypercholesterolemia. These patients have been notoriously difficult to treat, and current drug regimens including nicotinic acid and cholestyramine are often difficult for the patient to tolerate. This new protocol will examine the efficiency of the addition of neomycin as a hypocholesterolemic drug. Preliminary studies have reported that this drug is very well-tolerated and may be therapeutically useful. The current study should provide useful information on the possible therapeutic use of neomycin in the treatment of hypercholesterolemia.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02003-10 MDB

PERIOD COVERED
October 1, 1980 through September 30, 1981

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

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LAB/BRANCH
Molecular Disease Branch

SECTION
Peptide Chemistry

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TOTAL MANYEARS: 3.8	PROFESSIONAL: 1.8	OTHER: 2.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Research in our laboratory has focused on plasma lipoprotein and apolipoprotein (apo) composition and metabolism in normal and dyslipoproteinemic subjects. ApoA-I and apoA-II, the major protein constituents of human high density lipoproteins (HDL), have been extensively studied in terms of their site of production, plasma kinetics, and their relationship to various disease states. In addition a patient with an absence of apoA-I, a marked reduction in HDL, and premature coronary heart disease, has been identified, representing the discovery of a new disease entity. The interaction of apoA-I and apoA-II with plasma lipases is also being investigated. Ongoing studies of apoE metabolism support the concept that most patients with type III hyperlipoproteinemia (HLP) have an abnormal apoE. In addition, a patient with type III HLP associated with an absence of apoE has recently been found, and represents a new cause for this form of HLP. The effects of various diets on plasma lipoproteins and apolipoproteins are continuing projects under investigation.

PHS-6040
(Rev. 2-81)

Project Description

Objectives

1) To complete studies on apoA-I, apoA-II and apoE metabolism in normal and dyslipoproteinemic man.

2) To screen patients with various forms of dyslipoproteinemia for an absence or structural abnormality of apolipoproteins.

3) To determine the effects of various diets and drugs on plasma lipoproteins and apolipoproteins in normal and dyslipoproteinemic subjects and to study the clinical features of these latter subjects.

4) To study the interaction of apolipoproteins, various enzymes (lipoprotein lipase, hepatic triglyceride lipase) and plasma lipoproteins.

Methods Employed

Methodology for the isolation, quantitation, and radioiodination of plasma lipoproteins and apolipoproteins has been previously described. Methodology for plasma apolipoprotein determinations have been developed by other members of our branch. We have implemented a computerized record keeping system for purposes of storing and analyzing clinical and lipoprotein data on our patients.

Major Findings

1. Apolipoproteins (apo) A-I and A-II, the major protein constituents of human high density lipoproteins, are synthesized both by the liver and the intestine. The liver appears to release these apolipoproteins bound to lipids as lipoproteins in the HDL density region (1.063-1.21 g/ml), while the intestinal apoA-I and apoA-II enter plasma on both lymph chylomicrons and HDL. We have previously reported that lymph chylomicron apoA-I and apoA-II are rapidly transferred to HDL following entry of chylomicrons into plasma. Analysis of 24 hour thoracic duct lymph output in man are consistent with the concept that approximately 25-30% of apoA-I and apoA-II synthesis enters the plasma via lymph chylomicrons, with an additional 25% entering an intestinal HDL and the remaining 45-50% being contributed by the liver HDL (1).

2. As a result of analysis of data derived from 44 individual tracer studies performed in 22 normal subjects utilizing radioiodinated HDL, apoA-I, and apoA-II, a multicompartmental model for apoA-I and apoA-II has been developed (see Annual Report by Dr. Loren A. Zech). Results derived from these studies are consistent with the following concepts:

1) labeling of apoA-I and apoA-II as apolipoproteins or on HDL does not affect their specific radioactivity decay within HDL;

2) the mean residence time of apoA-I both in plasma and in HDL is significantly shorter than that of apoA-II;

3) the increased apoA-I levels seen in female subjects are due to increased apoA-I synthesis; and

4) the plasma apoA-I residence time which is inversely correlated with plasma triglyceride levels is an important determinant of apoA-I concentration in both males and females. Results of these studies have been submitted as a manuscript.

3. The effect of various apolipoproteins on the enzymic activity of lipoprotein lipase (LPL) and hepatic triglyceride lipase (HTGL) has been studied. ApoC-II, a protein constituent of human very low density lipoproteins (VLDL) and HDL, has previously been shown to activate LPL activity. LPL is necessary for triglyceride hydrolysis in plasma. ApoH, a protein constituent of human lymph chylomicrons, found in plasma in the lipoprotein free fraction (density > 1.21 g/ml) and also known as β_2-glycoprotein-1, has been shown to augment LPL activity 2-3 fold in the presence of apoC-II (2).

4. The function(s) of HTGL are as yet not fully elucidated. This enzyme appears to hydrolyze both triglyceride and phospholipids, and HTGL's most important role may be as a phospholipase. In this respect, HTGL may have a significant function in the breakdown of phospholipids on VLDL and HDL. Recently it has been shown that apoA-II, a major HDL protein constituent, activates HTGL enzymic activity (see Annual Repart of Dr. Claus Jahn, data submitted as manuscript).

5. Tangier disease, originally described by Fredrickson and associates, is a rare autosomal recessive disorder characterized by a markedly decreased HDL, abnormal triglyceride-rich lipoproteins, and cholesterol ester deposition in reticuloendothelial cells throughout the body, especially in the tonsils, lymph nodes and spleen. A review of all cases followed at the NIH, and those reported in the literature, indicates that Tangier patients may develop premature coronary heart disease but their disease is not nearly as striking or as premature as that seen in familial hypercholesterolemia (3). In addition, one Tangier homozygote was recently noted to have unusual bony deposits in the mandible (4).

6. Tangier homozygotes have apoA-I and apoA-II levels which are approximately 1% and 5% of normal, respectively. Other apolipoprotein levels are normal or only slightly reduced in these patients (5). We have previously reported that Tangier heterozygotes and homozygotes have a striking hypermetabolism of apoA-I and apoA-II which accounts for the reduced levels seen in the plasma of these patients. Ongoing studies in our branch are being carried out in an effort to determine the precise nature of the defect in Tangier disease.

7. The results of an HDL infusion study in Tangier homozygotes has been reported. Following HDL infusion, HDL_{2b} (density 1.063 - 1.10 g/ml) and HDL_{2a} (density, 1.10 - 1.125 g/ml) disappeared at a faster rate than HDL_3 (density, 1.125 - 1.21 g/ml). These alterations were associated with a more rapid disappearance of HDL apoA-I, cholesterol, and triglyceride than HDL apoA-II and phospholipid. This catabolic sequence of HDL constituents may have relevance for normal subjects as well (6).

8. Recently we have discovered a patient with severe coronary artery disease at age 45 years, corneal opacification, and a marked deficiency of plasms HDL. In contrast to homozygous Tangier disease, this patient had no detectable apoA-I in her plasma, and no evidence of cholesterol ester deposition in reticuloendothelial cells. We have named this entity apoA-I deficiency. Preliminary family studies suggest it has an autosomal recessive mode of inheritance.

9. The metabolism of apoE has been of great interest to members of our branch (see Annual Report of Dr. Richard Gregg). ApoE appears to be important in the receptor mediated uptake of chylomicron remnants by the liver. It has been shown that some patients with type III hyperlipoproteinemia (HLP) have an abnormal apoE pattern on isoelectric focusing. We have recently reported that type III HLP apoE is catabolized at a slower fractional catabolic rate on plasma lipoproteins than is normal apoE (7).

10. Type III HLP has been shown to be associated with premature coronary artery and peripheral vascular disease, tubo-eruptive or tendinous xanthomas, and the accumulation of cholesterol-rich very low density lipoproteins of abnormal electrophoretic mobility. Most patients with type III HLP have an abnormal apoE pattern on IEF as noted above. We have recently discovered a kindred affected with type III HLP where the affected family members have no detectable apoE in their plasma. These data are consistent with the concept that type III HLP may be due to an absence of apoE as well as an abnormal apoE.

11. The effect of exercise on plasma lipoproteins has been of interest, and was studied in normal volunteers while on an iso-weight balanced diet over a six week period. During this time a significant training effect was documented but no significant change in low density lipoprotein (LDL) or HDL cholesterol plasma concentrations was documented. These data are consistent with the concept that alterations in plasma lipoprotein concentrations seen with exercise may in large part be modulated by decreases in body weight (8).

12. It has been reported that hypercholesterolemic patients have enhanced platelet aggregability (especially in response to epinephrine) and decreased platelet survival, and it was suggested that these factors might play a role in the pathogenesis of the premature atherosclerosis seen in these patients. Over the past several years, in collaboration with Dr. Lawrence Corash, Hematology Department, Clinical Center, we studied platelet aggregation in heterozygous and homozygous familial hypercholesterolemic patients, as well as in normal subjects. Heterozygotes were older, had more diffuse and advanced atherosclerosis, and lower plasma cholesterol concentrations than did homozygotes. Homozygotes that were studied had only limited atherosclerosis. In these studies normals and homozygotes in general had normal platelet aggregation and survival, while heterozygotes have enhanced aggregation and shortened survival. These data are consistent with the view that platelet abnormalities seen in familial hypercholesterolemia are not related to plasma cholesterol concentrations but to the degree of atherosclerosis present (9).

13. Plasma lipoprotein abnormalities seen in patients with obstructive liver disease have been of interest to members of our branch. Patients with hepatoductular hypoplasia and severe obstruction of biliary flow have a marked inrease in LDL with the presence of lipoprotein X, and a decrease in HDL. These alterations can be somewhat ameliorated by cholestyramine administration (10). An extensive analysis of plasma lipoproteins and apolipoproteins in patients with obstructive liver disease due to primary cirrhosis has been carried out (see Annual Report of Dr. Claus Jahn).

14. The effect of alterations in dietary constituents on plasma lipoproteins has been an area of continuing interest. An increase in the ratio of polyunsaturated:saturated fats from 0.4 to around 2.0 results in reductions of both LDL and HDL cholesterol levels in both normal and hypercholesterolemic subjects (11). High carbohydrate (80% of total calories), very low fat, low cholesterol diets results in even greater decreases in both LDL and HDL cholesterol concentrations in normals and hypercholesterolemic patients (12). More recent studies carried out over the past 12 months indicate that an increased fat intake markedly potentiates the effect of dietary cholesterol in increasing plasma LDL cholesterol values.

Significance to Biomedical Research and the Program of the Institute

LDL and chylomicron remnants have been implicated in the pathogenesis of atherosclerosis, the leading cause of death in our society; while HDL may be a protective factor leading to a decrease in the amount of cholesterol deposition in the arterial wall. The factors which regulate the plasma concentrations of these various lipoproteins are, therefore, of great interest. ApoA-I and apoA-II are major protein constituents of HDL. Data derived from kinetic studies indicate that females have significantly higher apoA-I and apoA-II synthesis rates than do males, and this increased synthesis may be due to estrogen effects. The relationship between triglyceride-rich lipoproteins and HDL has been a topic of great interest to researchers in the lipoprotein field. As triglyceride-rich lipoproteins are catabolized, some of their lipid and protein constituents are transferred to HDL. In subjects with hypertriglyceridemia apoA-I and apoA-II fractional catabolic rates are enhanced, possibly because a larger fraction of these proteins are cleared with triglyceride-rich lipoproteins instead of HDL.

The finding of apoA-I absence as a cause of a marked reduction in HDL underscores the importance of this protein in HDL formation. The severe coronary heart disease (CHD) seen in the proband for this new disease entity reinforces the concept that a decreased HDL level is a risk factor for premature CHD.

The discovery that apoA-II is the activator for hepatic triglyceride lipase will hopefully lead to greater insight into the role of this enzyme in health and disease. There appears to be a significant amount of heterogeneity among HDL particles (13). For example, HDL containing apoE may have a significantly different function and metabolic rate as compared to HDL containing apoA-I and apoA-II. Recent data from our laboratory are consistent with the concept that type III HLP apoE is metabolized differently than normal apoE, and that apoE is important in the catabolism of chylomicron remnants. The discovery of apoE absence as a cause of type III HLP serves to underscore the functional importance of this apolipoprotein.

Since the use of platelet deaggregators has been suggested as being important in the treatment and prevention of atherosclerosis, we chose to evaluate platelet function in patients with familial hypercholesterolemia. These patients have been reported to have altered platelet function. Our data would suggest that the enhanced platelet aggregability and shortened platelet survival seen in familial hypercholesterolemia is a consequence of the atherosclerosis rather than the hypercholesterolemia. In this scheme, subjects with advanced atherosclerosis have enhanced platelet deposition and uptake, shortened platelet survival, and consequently younger, more metabolically active, and more aggregable platelets present in circulating plasma.

A knowledge of the effect of factors such as exercise and diet which affect plasma lipoproteins is crucial to our understanding of metabolism and our ability to treat patients with dyslipoproteinemia. Our own data are consistent with the concept that exercise effects (decreasing plasma triglycerides and VLDL, and increasing HDL) are at least in part modulated by weight reduction. Recently there has been a great deal of debate with regard to dietary alterations. Our own data are consistent with the concept that high cholesterol, high saturated fat diets increase plasma cholesterol significantly, mainly via an increase in LDL values.

Proposed Course

Continuing objectives include study of apolipoprotein (apoA-I, apoA-II, apoB, apoC-I, apoC-II, apoC-III, apoE, apoA-IV, apoH) in normal and dyslipoproteinemic subjects. We hope to define the genetics and lipoprotein metabolism in two new disease entities, apoA-I absence and apoE absence. We plan to further define the biochemical defect in Tangier disease. In addition, the patterns of inheritance and clinical features in dyslipoproteinemic patients, and modalities (diet and drug) whereby such patients can best be treated will be investigated.

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protein A-I and A-II deficiency (Tangier disease). Metabolism (in press, 1981).

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02010-10 MDB

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Structure and Function of Plasma Lipoproteins and Apolipoproteins

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

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	Rosemary Ronan, B.A.	Chemist	MDB	NHLBI
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COOPERATING UNITS (if any)

LAB/BRANCH

Molecular Disease Branch

SECTION

Peptide Chemistry

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.3	1.3	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)

$ApoA\text{-}I_{Tangier}$ and $apoA\text{-}II_{Tangier}$ were purified to homogeneity from a homozygous patient with Tangier disease. $ApoA\text{-}I_{Tangier}$ differed from apoA-I isolated from normal subjects in amino acid composition, apparent molecular weight on SDS gel electrophoresis, and heterogeneity of isoforms on two-dimensional polyacrylamide gel electrophoresis. $ApoA\text{-}II_{Tangier}$ was identical in physical-chemical properties to apoA-II from normal subjects. $ApoE_2$, E_3, and E_4 have been isolated in homogeneous forms and their physical-chemical, and metabolic properties analyzed. ApoH has been purified, and shown to be identical to β_2-glycoprotein-I. ApoH has a high content of cystine and proline, and unusual molecular properties due to its amino acid composition.

Human PTH (1-84) has been synthesized by the solid-phase method. The synthetic hormone had a similar biological activity and immunological properties to the native hormone.

PHS-6040
(Rev. 2-81)

Project Description

Objective:

1) Isolation and characterization of apoA-I and apoA-II from patients homozygous for Tangier Disease.

The methods for the isolation of plasma apolipoproteins from normal subjects have been detailed in previous reports. In addition the isolation of apoA-I from Tangier patients ($apoA\text{-}I_{Tangier}$) involved the fractionation of delipidation plasma apolipoproteins (density < 1.21 g/ml) on gel permeation chromatography followed by purification on a new preparative SDS gel electrophoresis system. The SDS system involved separation of partial purified apolipoproteins on a large gel (1.8 x 20 cm), and eluting the individual purified apolipoproteins from the gel by electrophoresis on the Isso Electrophoretic concentrator. ApoA-II from Tangier patients ($apoA\text{-}II_{Tangier}$) was isolated by ion exchange chromatography as has been described in previous reports.

Major Findings:

$ApoA\text{-}I_{Tangier}$ isolated from a homozygous patient with Tangier disease by gel permeation chromatography and preparative SDS gel electrophoresis was compared to apoA-I isolated in a similar manner from the plasma of normal donors. On Ouchterlony double immunodiffusion $apoA\text{-}I_{Tangier}$ and apoA-I from normal subjects showed a line of complete immunological identity. $ApoA\text{-}I_{Tangier}$ appeared to have a slightly greater apparent molecular weight than normal apoA-I on SDS gel electrophoresis. On analytical polyacrylamide gel electrophoresis (pH 8.9) in urea $apoA\text{-}I_{Tangier}$ appeared as a doublet whereas normal apoA-I migrated in the same position as principally a single band. On two dimensional electrophoresis $apoA\text{-}I_{Tangier}$ and apoA-I had a similar pH, however $apoA\text{-}I_{Tangier}$ had greater heterogeneity of the isoforms. The amino acid composition of $apoA\text{-}I_{Tangier}$ was consistently different from normal apoA-I.

$ApoA\text{-}II_{Tangier}$ was isolated to homogeneity by gel permeation followed by ion exchange chromatography. $ApoA\text{-}II_{Tangier}$ and normal apoA-II had a similar electrophoretic position on SDS gel electrophoresis, and polyacrylamide electrophoresis (pH 8.9) in 6 M urea. In addition two dimensional electrophoresis of $apoA\text{-}II_{Tangier}$ and apoA-II revealed an identical electrophoretic pattern. The amino acid compositions of $apoA\text{-}II_{Tangier}$ and normal apoA-II were virtually identical.

In summary $apoA\text{-}I_{Tangier}$ appears to be structurally different from apoA-I isolated from normal subjects. $ApoA\text{-}II_{Tangier}$ and normal apoA-II appear to be identical. These results are consistent with the concept that the molecular defect in Tangier disease is due to a structurally abnormal A-I apolipoprotein.

2) Isolation and characterization of apoE.

The methods for the isolation of plasma apolipoproteins have been detailed in previous reports. Heparin affinity chromatography followed by gel permeation chromatography on Sephacryl S-200 has been employed in the isolation of apoE.

Major Findings:

ApoE has now been isolated from patients homozygous for apolipoproteins E_2, E_3, and E_4. The apoE_2 patients characteristically have a Type III phenotype. ApoE_4 homozygotes have an increased frequency of the Type IV phenotype. ApoE_3 is the most common form of apoE, and has been isolated from a patient with Type IV hyperlipoproteinemia.

All the apoE proteins have characteristic patterns by isoelectric focusing, and single bands on SDS gel electrophoresis with a molecular weight of 32-34,000 by analytical ultracentrifugation. Of particular interest has been the metabolic studies carried out with the purified apoE_2 and apoE_4. ApoE_2 was shown to have a markedly delayed fractional catabolic rate when compared to apoE_4 consistent with the concept that Type III hyperlipidemia is due to a defect in the E apolipoprotein. This defect in apoE results in a decreased affinity for the liver E receptor resulting in the accumulation of chylomicron remnants, and the hyperlipidemia characteristic of the Type III phenotype.

3) Isolation and characterization of apoB from plasma and thoracic duct lymph.

Methods Employed:

Two separate apolipoproteins of high molecular weight designated apoB-I or large apoB and apoB-II or small apoB have been identified in human plasma and thoracic duct lymph. ApoB-I is the major protein in LDL, and can be isolated directly by delipidation of LDL. ApoB-II is a major protein in lymph chylomicrons and is isolated by preparative SDS gel electrophoresis from delipidated human thoracic duct lymph.

Major Findings:

ApoB-I and apoB-II have been isolated in homogeneous form. ApoB-I and apoB-II have an apparent molecular weight by SDS gel electrophoresis of approximately 300,000 and 180,000 respectively. The amino acid analyses of apoB-I and apoB-II are distinctly different consistent with the view that these two proteins are separate, unique apolipoproteins. Antibodies have been prepared against apoB-I and apoB-II in rabbits. The antibodies against B-I and B-II are distinct, and show no cross reactivity. The evidence thus far collected is consistent with the view that these two apolipoproteins are separate unique proteins and not oligomeric subunits or fragments of a large single protein. Detailed characterization and analysis of these two apolipoproteins is underway.

4) Isolation and characterization of apoH.

ApoH was isolated from the VLDL of normal subjects, patients with Type V hyperlipidemia, and from human thoracic duct lymph. ApoH was isolated by heparin affinity chromatography followed by Sephacryl S-200 gel filtration chromatography.

Major Findings:

ApoH isolated from human plasma or thoracic duct lymph was a single ban on SDS gel electrophoresis. On isoelectric focusing apoH was characterized by several bands. ApoH contains a high mole percentage of cystine and proline residues by amino acid analysis. On reduction of the disulfide bonds by dithiothreitol there is a marked change in the electrophoretic migration of apoH on SDS gel electrophoresis. These findings suggest that apoH may contain a high degree of ordered-structure due to the high frequency of cystine and proline in the amino acid sequence of the protein. Detailed physical-chemical studies of apoH are currently in progress.

Antibodies to apoH have been produced in goats, and a radial immunodiffusion assay for apoH in under development. ApoH will be quantitated in normals, and in patients with hypertriglyceridemia. The functional role of apoH is activating lipoprotein lipase was detailed in last years annual report. Two apolipoproteins C-II and H appear to act as cofactors for lipoprotein lipase in triglyceride metabolism. Defects in the function of apoH would be anticipated to be associated with hypertriglyceridemia.

5) Solid Phase Synthesis of Human Parathyroid Hormone.

Human parathyroid hormone (h-PTH), an 84 amino acid polypeptide, was synthesized by the Merrifield solid phase technique using the phenylacetamidomethyl (PAM) resin. The PAM resin was chosen since it has increased acid stability, and the insertion of the PAM group between the peptide and the polystyrene matrix reduces the rate of peptide loss from the resin to only 1% of that of conventional styrene-divenylbenzene resins and enhances the applicability of the PAM resin for synthesizing longer proteins t-BUC groups were used to protect the α-amino groups of all amino acids except arginine where the more soluble amyloxy derivative was employed. Initially a 25% solution of TFA in CH_2Cl_2 was used for deprotecting the α-amino group. The concentration was increased to 40% by step 40. Amino acids were attached to the peptide-resin by coupling with dicyclohexyl carbodimide (DCC) and the t-BOC-amino acid. The active ester method was used to couple the p-nitrophenyl esters of asparagine and glutamine. The protected peptide was cleared from the resin by anhydrous hydrogen fluoride. The synthetic h-PTA was purified by gel filtration followed by ion exchange chromatography.

Major Findings:

Synthetic h-PTH purified by gel and ion exchange chromatography gave a single band on polyacrylamide gel electrophoresis (pH 4.4). The amino acid composition of the purified hormone was similar to the native h-PTH isolated from parathyroid adenomas. Analysis of the synthetic hormone by Edman degredation revealed no significant overlaps, or deletions. The biological activity of the purified hormone was virtually identical to the native hormone. Thus the intact 1-84 human hormone has been synthesized in a fully biologically active form. The availibility of the synthetic hormone will permit detailed biological and physiological studies on human PTH. In addition the synthetic h-PTH will provide material for the development of radial immunoassays for h-PTH for the diagnosis and clinical study of patients with clinical disorders of calcium metabolism.

Significance to Biomedical Research and the Program of the Institute:

The isolation, characterization, and sequence analysis of the human plasma apolipoproteins is an ongoing program designed to ultimately understand the physiological role and molecular mechanisms involved in the synthesis, transport, and metabolism of plasma lipoproteins in normal individuals and patients with disorders of lipid transport and atherosclerosis.

Proposed Course:

The isolation, characterization, and structural analysis of plasma apolipoproteins within human thoracic duct lymph and plasma will be continued. Major emphasis will be continued on apolipoproteins H, E, B-I and B-II. The ultimate understanding of the molecular defect in apoE in type III patients will be of major importance in our understanding of the role of apoE in lipid metabolism. The identification of the defect in apoA-I in Tangier disease will be pursued with the ultimate aim of determining the amino acid sequence of the protein. The identification of apoH as a cofactor in the modulation of the enzymic activity of lipoprotein lipase will provide new insights into the regulation of triglyceride metabolism. Several fragments as well as intact apolipoproteins will be prepared by solid phase synthesis. This will permit the elucidation of the structure-function requirements for lipid binding and cofactor function of the plasma apolipoproteins. The continued elucidation of the structure and function of plasma apolipoproteins will be a prerequisite to our ultimate understanding of the molecular mechanisms involved in lipid transport and metabolism.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02011-06 MDB

PERIOD COVERED October 1, 1980 through September 30, 1981

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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Molecular Disease Branch

SECTION

Peptide Chemistry

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NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.0	2.0	4.0

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 projects are directed towards a greater understanding of the quaternary organization of plasma lipoproteins and of the function of the oligomeric species involved in the transport and metabolism of lipids in plasma. The apolipoprotein composition of plasma lipoproteins is viewed as the governing factor in directing lipoprotein metabolism. Specificity is believed to be related directly to apolipoprotein secondary, tertiary and quaternary structure. Studies of the hydrodynamic properties of apolipoproteins have been extended to include apoH and the mixed interaction between two self-associating apolipoproteins, apoA-I and apoC-I. A method of analyzing irreversible ligand-cell interactions has been developed and applied to plasma lipoprotein cell interactions. The molecular properties of radiolabeled apoA-I have also been evaluated. A knowledge of the molecular properties of the specific self-associated and mixed-associated oligomeric apolipoprotein complexes (modified and native) are of major importance to our ultimate understanding of plasma lipoprotein metabolism.

PHS-6040
(Rev. 2-81)

Project Description

Objective:

1) 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 apolipoprotein and lipid composition of plasma lipoproteins is not static; distribution changes with metabolism and depends critically upon the composition and concentration of other components of plasma. Since changes in the apolipoprotein composition of plasma lipoproteins are necessarily governed by laws of mass action, their behavior is most easily addressed by a comparison of the rates of the interactions involved in lipid metabolism. The simplest case is one in which the rate of dissociation of an apolipoprotein from a lipid-apolipoprotein complex is slow so as to be essentially irreversable for most studies. Apolipoproteins in this category only slowly, if at all, undergo exchange and transfer reactions and thus any redistribution represents net transfer. ApoB is the only known apolipoprotein in this category. All other known apolipoproteins are believed to belong to the second category, in which molecular interactions are freely reversible. In this category the apolipoprotein composition of plasma lipoproteins is governed through the laws of mass action by the affinity of specific apolipoprotein and/or lipoprotein species for different lipoprotein particles. With metabolism the lipoprotein particles are modified covalently, changing their affinity for specific apolipoprotein and/or lipoprotein complexes, which results in a redistribution of apolipoproteins. The role that lipid-free species play in these exchange/transfer processes is not known. In order to quantitate these interactions we have undertaken a systematic investigation of the solution properties of apolipoproteins. Previous reports have stressed the self-association of apolipoproteins in aqueous solution with particular emphasis on the major conformational changes that are concomitant with association. Apolipoproteins also undergo specific mixed-associations with other apolipoproteins. The analysis of mixed-associations between two self-associating proteins is simplified greatly if the spectral properties of the two apolipoproteins are different from one another. During the past year we have evaluated the mixed interaction between apoA-I and apoC-I using sulfenylated apoC-I.

Methods Employed:

Secondary structure, i.e., α-helical, β-pleated sheet, and random configurations, was investigated by using a Cary model 61 spectrometer. Tertiary structure, i.e., the mode of intramolecular folding, was investigated by monitoring changes in the fluorescence and absorption properties on a Perkin-Elmer Model MPF-4 fluorescence spectrophotometer and a Beckman Acta 3 spectrophotometer. Quaternary structure, i.e., intermolecular protein interaction, was studied by molecular weight measurements with a Spinco Model E ultracentrifuge and a Chromatrix Laser Light Scattering Photometer. Calculations and least square fits of the data to various models were performed with the MLAB program on the DEC system 10 computer at NIH.

Major Findings:

As indicated in last years annual report, analysis of mixed association between two self-associating systems is simplified greatly if one of the proteins contains a chromophore that absorbs in the visible range. ApoC-I was sulfenylated with sulfenyl chloride. The absorption spectra of the modified species (S-apoC-I) was "red shifted" well above 300 nm with a maximum at 365 nm. All other molecular properties, i.e., circular dichoric spectra and molecular weight dependence upon concentration and immunoreactivity, were indistinguishable from native apoC-I.

Sedimentation equilibrium measurements of S-apoC-I in the presence and absence of apoA-I were obtained at rotor speeds of 12,000, 15,000, 18,000, 22,000 and 26,000 RPM. The profile of concentration versus radius at equilibrium was obtained by absorption measurements at 365 nm, a comparison of data in the presence and absence of apoA-I is a sensitive measure of mixed-association. Complex formation between S-apoC-I and apoA-I was detected easily at all rotor speeds; the apparent molecular weight of S-apoC-I was increased substantially in the presence of apoA-I. A least squares analysis of data at all rotor speeds was most consistent with a single mixed oligomer containing 2 molecules of apoA-I and 4 molecules of S-apoC-I.

With this data, and that obtained by our laboratory over the past several years, self- and mixed-association between apolipoproteins can be summarized as follows:

Self-Association

Apolipoprotein	Association Scheme
apoA-II	monomer-dimer
reduced and carboxymethylated apoA-II	monomer-dimer
apoA-I	monomer-dimer-tetramer-octamer
apoC-I	monomer-dimer-tetramer
apoC-III	monomer-dimer-trimer

Mixed-Association

Apolipoproteins	Oligomer Stoichiometry
apoA-I:apoA-II	1:1, 2:2
apoA-II:apoC-I	2:4
apoA-I:apoC-I	2:4

Objective:

2) Evaluation of the role of apolipoproteins in the quaternary organization of HDL.

Chylomicrons and very low density lipoproteins (VLDL), secreted by the intestine and liver respectively, are large lipid-protein complexes that are involved in the transport and metabolism of triglycerides, cholesterol and cholesterol esters. The nacent particles, which are believed to be composed primarily of apolipoprotein B and lipids, gain an additional complement of apolipoproteins upon entering the plasma. Thus, the distribution of apolipoproteins A-I, A-II, C-I, C-II, C-III_2 and E are dependent, through the laws of mass action, on the concentration of chylomicrons and VLDL in plasma. The role of each of these apolipoproteins in metabolism is not known with certainty; apolipoproteins B and E are believed to serve as recognition sites for the specific uptake of plasma lipoproteins by cells, apoC-II is believed to serve as an activator of lipoprotein lipase, and apoA-I activates lecithin cholesterol acyltransferase. It has recently been established that an additional protein, β_2-glycoprotein-I (β_2GI) is isolatable with plasma lipoproteins. β_2GI, which is approximately 18% by weight carbohydrate, was described originally by Schultye, Heide and Haupt in 1961, and preliminary physical properties were reported by Finleyson and Mushinski in 1967.

Approximately 16% by weight of plasma β_2GI is found with chylomicrons and VLDL, 2% with low density lipoproteins (LDL), 17% with high density lipoproteins (HDL) and the remainder (65%) in the 1.21 density infranate. It is also known that selective precipitation of triglyceride rich lipoproteins by sodium laural sulfate or sodium dodecyl sulfate requires the presence of β_2GI. We have recently found that β_2GI activates lipoprotein lipase in the hydrolysis of triglyceride-phospholipid emulsions. Since β_2GI is isolatable on plasma lipoproteins, has a high affinity for triglyceride-rich lipoprotein particles, and activates lipoprotein lipase we have designated β_2GI as apoH.

In view of these recent findings we have begun a systematic investigation of the interaction(s) between apoH and plasma lipoproteins. A prerequisite to these studies is a more complete understanding of the molecular properties of the isolated apolipoprotein. During the past year we have evaluated the molecular weight, circulár dichroic spectra and surface activity of apoH.

Methods Employed:

Secondary structure, i.e., α-helical, β-pleated sheet, and random configurations, was investigated by using a Cary model 61 spectropolarimeter. Tertiary structure, i.e., the mode of intramolecular folding, was investigated by monitoring changes in the fluorescence and absorption properties on a Perkin-Elmer Model MPF-4 fluorescence spectrophotometer and a Beckman Acta 3 spectrophotometer. Quaternary structure, i.e., intermolecular protein interaction, was studied by molecular weight measurements with a Spinco Model E ultracentrifuge and a Chromatrix Laser Light Scattering Photometer. The drop-volume method was used for surface tension measurements. The apparatus consisted of a 1 ml Hamilton syringe attached to a metal cyclindrical tip of known diameter. 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:

Previous estimates of the molecular weight of apoH have ranged from 40,000 to 100,000. In the present study sedimentation equilibrium measurements resulted in a molecular weight of 43,400 $\pm$ 500 in non-denaturing buffers and 42,600 $\pm$ 500 in guanidinium chloride. The circular dichroic spectrum of apoH is unusual for a typical gobular protein: there is no strong maximum near 200 nm and no major transitions in the 208-222 nm region. The major features of the spectrum include a positive maximum at 235 nm and a weak negative maximum at 205 nm. The positive maximum is lost with increasing concentrations of quanidinium chloride. There is an initial plateau region below 2M GdmCl and the transition is complete by 3M GdmCl.

The profile of surface tension (γ) versus concentration of protein was obtained for apoH, apoA-I and chymotrypsinogen. The initial slope of γ versus apoA-I concentration is much greater than that observed for chymotrypsinogen or apoH, and the surface pressure ($\pi o-\pi$) at higher concentrations of protein appears to be much higher for apoA-I. These data represent the surface tension at one minute and the differences observed may be due to differences in rate or equilibrium surface pressure.

Both the rate of absorption and the equilibrium surface pressure were greater for apoA-I. The initial slopes are monoexponential and correspond to rate constants of 2.55×10^{-2} sec $^{-1}$ and 9.68×10^{-3} sec^{-1} respectively for apoH and apoA-I.

The rate of absorption of apoH is increased dramatically in acidic and basic solutions. This change in rate does not, however, result in a change in the equilibrium surface pressure. Acidification also results in a change in the circular dichroic spectra of apoH. The positive maximum at 235 nm decreases slightly in magnitude and there is a more dramatic increase in negative intensity at 202nm. The positive maximum at 235 nm is unchanged from pH 7.4 to 11.0; however, there is a decrease in magnitude in the negative maximum and a slight shift to longer wavelengths at pH 11.0.

In summary apoH is a single polypeptide chain of molecular weight 43,000. The circular dichroic spectra in the far ultraviolet is unusual with a weak maximum at 230 nm and a relatively weak minimum at 205 nm. At neutral pH the affinity of apoH for an air-water interface is comparable to gobular proteins. Treatment with acid or base, although causing only minor changes in circular dichroic spectra, results in major increases in affinity for air-water interfaces such that apoH resembles the more flexible apolipoproteins (e.g., apoA-I, apoA-II, and apoC-I) under these conditions. Present studies are directed towards the affects of lipid on the secondary and tertiary folding of this unique apolipoprotein.

Objective:

3) Evaluation of the molecular properties of radiolabeled apolipoproteins.

Several laboratories, including the Molecular Disease Branch, have investigated the kinetics of the in vivo metabolism of radiolabeled apolipoproteins. The value of these data in terms of the metabolic fate of native apolipoproteins and lipoprotein particles depends directly upon any perturbations in the molecular properties of the resulting radiolabeled plasma lipoproteins caused by the labeling and/or incubation (recombination) procedure employed. The labeled apolipoprotein must mimic the distribution of the unlabeled species. We have begun an intensive comparison of the molecular properties of radiolabeled and unlabeled apolipoproteins A-I and A-II. In last years annual report we summarized our studies of the behavior of radiolabeled apoA-I on column chromatography. Briefly, at low initial concentrations where the elution profile favors monomeric species (native apoA-I self-associates according to a monomer-dimer-tetramer-octamer scheme) the peak of absorbancy preceded the corresponding peak of radioactivity. In contrast at high initial concentrations where octameric apoA-I is favored the elution profiles of the faster migrating components as obtained by radioactivity and absorbancy measurements, overlap one another. These combined results can be accounted for by three possible mechanisms:

1) Non-specific interaction between monomeric iodinated apoA-I and the column matrix. In this case the molecular properties of iodinated apoA-I would be the same as native apoA-I.

2) Iodination results in a decrease in the equilibrium constant for oligomer formation. In this case all labeled apolipoproteins participate in self-association with unlabeled apolipoproteins, but the specific activity is higher in the monomeric species which results in a shift of the radioactivity profile to favor slower migrating components.

3) Iodination of apoA-I results in the formation of an incompetent monomer due to either double labeling of a single protein molecule or the labeling of a specific tyrosine residue. This incompetent monomer does not participate in the self-association, which results in a higher specific activity for slower migrating components.

Over the past year we have evaluated these three possibilities.

Methods Employed:

Secondary structure, i.e., α-helical, β-pleated sheet, and random configurations, was investigated by using a Cary model 61 spectropolarimeter. Tertiary structure, i.e., the mode of intramolecular folding, was investigated by monitoring changes in the fluorescence and absorption properties on a Perkin-Elmer Model MPF-4 fluorescence spectrophotometer and a Beckman Acta 3 spectrophotometer. Quaternary structure, i.e., intermolecular protein interaction, was studied by molecular weight measurements with a Spinco Model E ultracentrifuge and a Chromatrix Laser Light Scattering Photometer. Calculations and least square fits of the data to various models were performed with the MLAB program on the DEC system 10 computer at NIH.

Major Findings:

Radiolabeled apoA-I was fractionated into two pools by column chromatography. The first fraction (pool 1) was taken from that portion of the profile that correspond to octameric apoA-I and the second fraction was taken from that portion of the profile that correspond to monomeric apoA-I. The molecular properties of these pools were evaluated by circular dichroic measurements, sedimentation equilibrium, and immunochemical analysis by radialimmunodiffusion.

At high concentrations of protein the circular dichroic spectra of both pools indicated a high helical content and were quite similar to that previously found for native apoA-I. With decreasing protein concentration the mean residue ellipticity of pool 1 at 220 nm decreased by approximately 22%, again in accordance with that predicted for native apoA-I. In contrast, the mean residue ellipticity of pool 2 at 220 nm decreased by over 50% with dilution. The weight average molecular weight of pool 1, as obtained by equilibrium sedimentation, increased with increasing protein concentration as predicted from previous studies with native apoA-I (M_r = 37,000 at 0.1 mg/ml increasing to 98,000 at 0.6 mg/ml). In contrast the molecular weight of pool 2 increased only slightly over this concentration range (M_r = 26,000 @ 0.2 mg/ml to 30,000 @ 0.6 mg/ml). Finally, quantitation of the protein in pool 1 using an antibody raised against native apoA-I by radialimmunodiffusion (RID) correspond to concentration based on optical density measurements (0.092 mg/ml by E_{280} versus 0.108 mg/ml by RID). Results of RID quantitation of pool 2 were much lower than that predicted by absorbancy measurements (0.094 mg/ml by E_{280} versus 0.029 mg/ml by RID).

For the first two mechanisms the molecular properties of pools 1 and 2 would be indistinguishable from one another. The combined results of circular dichroic measurements, immunochemical quantitation and sedimentation equilibrium demonstrate experimentally that pools 1 and 2 are not the same; pool 1 is very similar to native apoA-I where as pool 2 favors monomeric apoA-I. Mechanism (3) above is the most consistent with all experimental data. The molecular properties of unfractionated radiolabeled apolipoprotein A-I are not the same as native apoA-I. This may have major consequences in the interpretation of in vivo kinetic studies, and in the quantitation of apoA-I by radioimmunoassay.

Objective:

4) Evaluation of irreversable-liquid-cell interactions: differentiation between binding and internalization.

The initial event in the biological response of cells to numerous ligands, ranging from large polypeptides, such as the glycoprotein hormones, to small organic molecules, such as amino acids and opiates, is believed to be an interaction between the ligand and a cell surface receptor. Correlation of this event with subsequent enzymatic and biochemical responses by the cell is quite complex; responses range from relatively minor changes in membrane permeability to intricate regulation of the adenylate cyclase system. Fundamental to understanding this process is a knowledge of the affinity and mechanism of ligand - receptor interactions. Cell surface receptors have proven to be quite difficult to purify and isolate in a biologically active form and most binding studies have involved

direct measurement using intact cells. The analysis of this data poses special problems, expecially when the ligand-receptor is internalized and/or degraded during the course of experimental observations. These complications can be minimized by appropriate experimental design and data analysis.

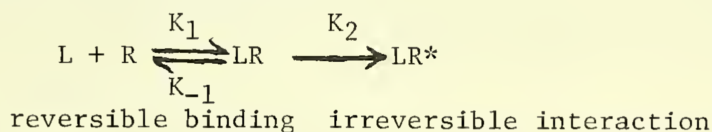
Major Findings:

For purposes of the present treatment we shall divide ligand cell interactions into two categories; those that result in (1) reversible and those that result in (2) irreversible ligand - receptor binding.

The majority of cell effectors that have been well characterized are believed to fall into the first category. In most of these systems reversible receptor - ligand interactions lead ultimately to the generation of a "second messenger", calcium and cAMP are implicated most frequently, which is responsible for carrying the signal into the cell and triggering the appropriate biological response. Analysis of the affinity and mechanism of binding for this category is straight forward; most investigations have involved measurements of binding at equilibrium with subsequent analysis by classical treatments such as that proposed by Scatchard.

Some well characterized systems fall into the second category; the most notable being low density lipoproteins (LDL) and certain neurotoxic polypeptides isolated from snake venom. Irreversible interactions can also result from covalent modification of the ligand upon binding and/or internalization (with or without the corresponding receptor) during the time course of the experimental observations. These systems are best analyzed by following the kinetics of ligand - cell interactions. In the present manuscript we develop a kinetic treatment for ligand - cell interactions and discuss the possibility of obtaining the relative affinity and total number of receptors for systems exhibiting irreversible interactions.

The simplest treatment of transport is to follow reversible binding with an irreversible interaction:



$$L + R \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} LR \xrightarrow{K_2} LR^*$$

reversible binding irreversible interaction

Where K_2 is the rate constant for irreversible interaction. From the conservation of mass:

$$R_t = R + LR + LR^*$$

The rate of irreversible interaction, I, is given by

$$I = K_2[LR]$$

Combining equations (12) and (13) one obtains:

$$\frac{I}{R_t} = \frac{K_2[LR]}{[R] + [LR] \quad [LR^*]}$$

For the laws of mass action and steady state assumptions:

$$[LR] = \frac{K_1}{K_{-1} + K_2} [L][R] = K'[L][R]: \quad \text{where } K' = \frac{K_1}{K_{-1} + K_2}$$

$$[LR^*] = K_2[LR] = K_2K'[L][R]$$

Thus from equations (14), (15) and (16):

$$\frac{I}{R_t} = \frac{K_2K' [R][L]}{[R] + K'[R][L] + K_2K'[R][L]}$$

Simplifying:

$$\frac{I}{R_t} = \frac{K_2K' [L]}{1 + K' [L] + K_2K' [L]}$$

$$\frac{I}{R_t} = \frac{K_2K' [L]}{1 + K' [L] (1 + K_2)}$$

$$\frac{I}{[L]} = K'K_2R_t - IK' (1 + K_2)$$

For the case where the amount of receptor-ligand complex in the irreversible stage is small compared to the total amount of receptor ($R + LR >> LR^*$), i.e. for initial rates, the above equation reduces to:

$$\frac{I}{L} = K'K_2R_t - IK'$$

This equation is linear and the slope of a plot of I/L versus L yields K'. In the case where the rate of ligand dissociation from the cell is much greater than the rate of internalization, i.e., $K_{-1} >> K_2$, K' corresponds to the equilibrium constant for reversible ligand-cell interactions.

Thus for complex systems where reversible binding is followed by an irreversible step, such as internalization, one can, in principle, determine the equilibrium constant by measuring the reversible or irreversible interactions. The number of sites, however, cannot be determined by measuring only the irreversible interactions; the terms K_2 and R_t, defined above, cannot be separated mathematically. The intercept of a Scatchard type of analysis for irreversible systems is the product of the number of binding sites and the rate of internalization.

With this treatment one can, in theory, analyze binding and internalization processes for complex systems by treating irreversible interactions as the first phase of internalization. The required data are initial rates of irreversible interaction at several different initial concentrations of ligand. There are few data in the literature that are complete enough to apply the above treatment. One of the more comprehensive studies as a function of time that is that of Pitas et al., (PNAS 76: 2311-2315, 1979) on the interaction between plasma lipoproteins, LDL and LpE, and human fibroblasts. We have analyzed that data using the above treatment. For both lipoproteins plots of rate/(lipoprotein concentration) versus rate are non-linear. This is especially apparent for LpE. Plots of rate versus lipoprotein concentration are sigmodial, resembling that expected for positively cooperative systems. Based on a molecular weight of 3.6×10^6 for LpE there are approximately 16 molecules for apoE per lipoprotein particle. In addition, Pitas et al., (J. Biol. Chem. 255: 5454-5460, 1980) have concluded that LpE binds to multiple receptors on fibroblast membranes. Thus all available data are consistent with positively cooperative interactions between apoE-HDL_c and membrane receptors.

Objective:

5) Automation of data collection and analysis for the model E ultracentrifuge.

The method of choice for measuring the molecular weight of macromolecules is sedimentation equilibrium using the model E ultracentrifuge. This technique does not require calibration with molecules of known molecular weight and the results do not depend on size, charge or shape. Currently our model E is in continuous operation in order to satisfy our own as well as other NHLBI investigators requirements for molecular weight determination. Results are currently hand calculated, which is the rate limiting step in data collection and analysis. An automatic method of collecting data would minimize operator error and increase greatly the efficiency of my laboratory.

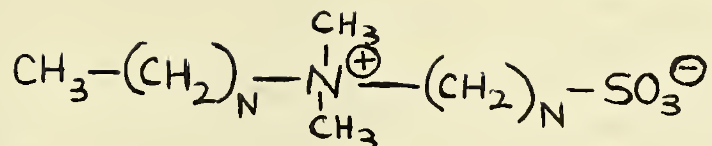
Major Findings:

In collaboration with Ramon L. Tate and Arthur R. Schultz, Jr. (DCRT) the model E ultracentrifuge was interfaced with a PDP 11/03 microcomputer. The system supervises the acquisition, display and preprocessing of data from the ultracentrifuge and is capable of interacting with the DEC system-10 facilities on campus. Current programs on the DEC system-10 allow automatic molecular weight calculations for non-interacting as well as interacting macromolecules. The combined system is efficient and minimizes operator error in molecular weight analysis.

Objective:

6) Evaluation of the molecular properties of a new series of detergents: sulfobetaine derivatives of bile acids.

N-alkyl sulfobetaines which have the following general structure:



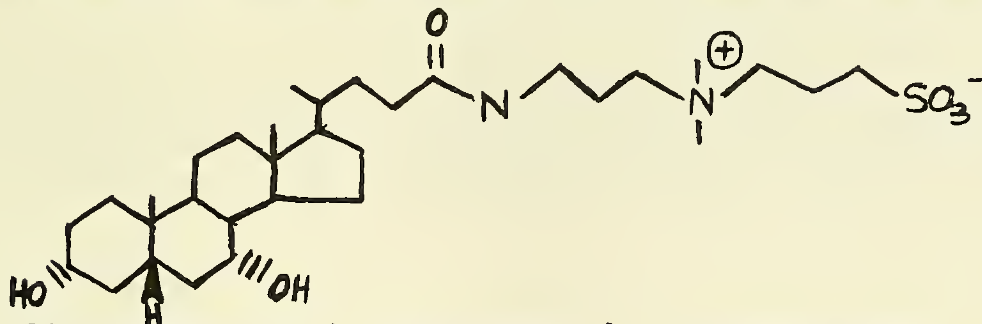
have many of the properties required of an ideal detergent for solubilizing biological compounds. This series of surfactants does not migrate in electric fields, bind to ion-exchange resins, conduct electricity, or contribute to ionic strength and can be synthesized and purified to homogeneity easily. However, these compounds are such strong detergents that solubilization usually involves irreversible denaturation. A new series of detergents based on bile acid derivatives of sulfobetaines have been synthesized recently by Leonard M. Hjelmeland (DPB, NICHHD). This series of detergents have many of the properties of n-alkyl sulfobetaines but are much "milder" detergents for biological systems. In order to characterize more fully these unique surfactants we have investigated their physical properties and interactions with proteins over the past year.

Methods Employed:

Secondary structure, i.e., α-helical, β-pleated sheet, and random configurations, was investigated by using a Cary model 61 spectropolarimeter. Tertiary structure, i.e., the mode of intramolecular folding, was investigated by monitoring changes in the fluorescence and absorption properties on a Perkin-Elmer Model MPF-4 fluorescence spectrophotometer and a Beckman Acta 3 spectrophotometer. Quaternary structure, i.e., intermolecular protein interactions, was studied by molecular weight measurements with a Spinco Model E ultracentrifuge and a Chromatrix Laser Light Scattering Photometer. The drop-volume method was used for surface tension measurements. The apparatus consisted of a 1 ml Hamilton syringe attached to a metal cylindrical tip of known diameter. Calculations and least square fits of the data to various models were performed with the MLAB program on the DEC system 10 computer at NIH.

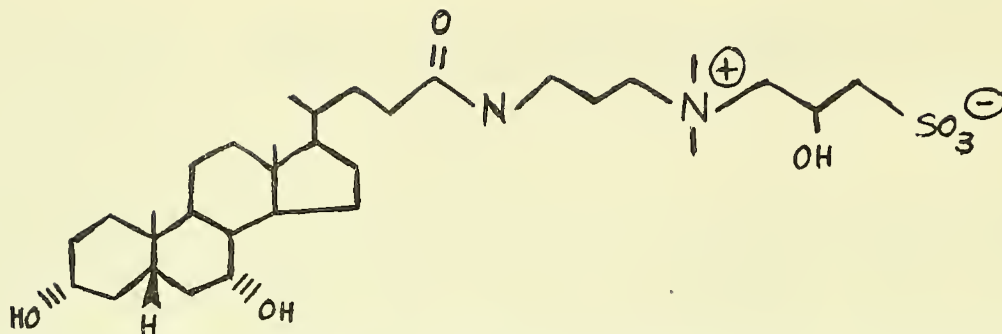
Major Findings:

The compounds tested and suggested trivial names are given below:



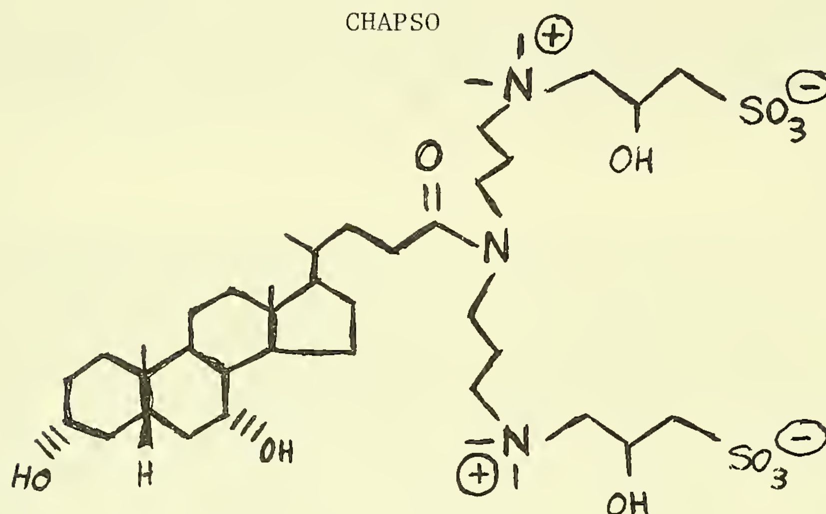
3-[(3-Cholamidopropyl) Dimethylammonio]-1-Propanesulfonate

CHAPS



3-[(3-cholamidopropyl) Dimethylammonio]-2-Hydroxy-1-Propane sulfonate

CHAPSO



3,3'[(Choloyimino) Bis [Trimethylene (Dimethylimino)]]
Bis[2-hydroxy-1-Propanesulfonate]

BIS CHAPSO

Each of these compounds do not have a high absorbancy in the near ultraviolet ($E_{280\ nm}$ = 0.03) or exhibit a circular dichroic spectra in the far ultraviolet region, and are thus well suited for optical studies with macromolecules. Critical micellular concentrations (CMC), partial specific volumes, and aggregation numbers are listed in table 1.

TABLE 1

Compound	Molecular Weight	CMC (mM)	V (ml/g)	Aggregation number
CHAPS	615	8	0.79	10
CHAPSO	631	8	0.74	10
BIS CHAPSO	854	9	0.75	15

CHAPS was found to be an effective solubilizing agent for cytochrome P-450. Species with molecular weights as low as 114,000 were apparent by sedimentation equilibrium measurements; previous attempts at solubilizing this protein with other detergents yielded lower molecular weights of 300,000. Chaps also caused a monotonic increase in the mean residue ellipticity of apolipoprotein C-III_2 at low concentrations (within the CMC region, i.e., below 15 mM) and thus interacts well with hydrophobic proteins. In summary this series of detergents seems well suited for solubilization of membrane bound proteins and macromolecules.

Significance to Biomedical Research and Program of the Institute:

These investigations are directed toward a greater understanding of the quaternary organization and function of the plasma lipoproteins. The plasma lipoproteins are complex with structures that are sensitive to numerous perturbations including pressure, ionic strength and temperature. The apolipoprotein and lipid composition of plasma lipoproteins is related directly to the concentration and composition of other components of plasma, including other plasma lipoproteins. A quantitative knowledge of the organization and interactions of plasma lipoproteins is fundamental to our understanding of lipid transport and metabolism in normal individuals and in patients with disorders of lipid metabolism and atherosclerosis.

Proposed Course:

Investigations concerning the molecular properties of apolipoproteins will be expanded to include apoA-IV and studies on apoE and mixed-associations between apolipoproteins will be continued. In addition, studies on the molecular properties of HMG-CoA reductase and lipoprotein lipase shall be initiated. These studies continue to form a firm framework for our ultimate understanding of *in vivo* plasma lipoprotein interactions.

Publications:

1. Osborne, J. C., Jr.: Evaluation of the mixed association between two self-associating systems by sedimentation equilibrium, (1980) Biochim. Biophys. Acta, 619, 567-571.

2. Osborne, J. C., Jr., Brewer, H. B., Jr.: Solution properties of the plasma apolipoproteins (1980). Annals of The New York Academy of Science, 348, 104-121.

3. Osborne, J. C., Jr., Servillo, L.: Equilibrium sedimentation of interacting systems: Mixed interaction between two self-associating proteins (1981). Biophysical Chemistry, 13.

4. Servillo, L., Brewer, H. B., Jr. and Osborne, J. C., Jr.: Evaluation of the mixed interaction between apolipoproteins A-II and C-I by equilibrium sedimentation (1981). Biophysical Chemistry, 13.

5. Osborne, J. C., Jr., Powell, G. M. and Brewer, H. B., Jr.: Analysis of the mixed association between human apolipoprotein A-I and A-II in aqueous solution (1980). Biochim. Biophys. Acta, 619, 559-567.

6. Osborne, J. C., Jr., Moss, J., Fishman, P. H., Nakaya, S. and Robertson, D. C.: Specificity in protein-membrane associations: The interaction of gangliosides with E. coli heat-labile enterotoxin and choleragen (1981). Biophysical Journal, in press.

7. Moss, J., Osborne, J. C., Jr., Fishman, P. H., Nakaya, S. and Robertson, D. C.: Escherichia coli heat-labile enterotoxin: Interaction with gangliosides and ADP-Ribosyltransferace activity. In Proceeding of the Sixteenth Joint Conference on Cholera, Gifu City, Japan.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02012-06 MDB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Regulation of Rat and Human liver 3-hydroxy-3-methylglutaryl Coenzyme A Reductase by Reversible Phosphorylation

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

PI:	Zafarul H. Beg, Ph.D.	Senior Investigator	MDB	NHLBI
Others:	John H. Stonik, B.S.	Chemist	MDB	NHLBI
	H. Bryan Brewer, Jr., M.D.	Chief	MDB	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Molecular Disease Branch

SECTION
Peptide Chemistry

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	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)

Microsomal 3-hydroxy-3-methlyglutaryl coenzyme A reductase (HMGR) of human liver was inactivated in vitro by ATP-Mg in a time dependent manner; this inactivation was mediated by reductase kinase (RK). Polyacrylamide gel electrophorisis of purified HMGR incubated with RK and [^{32}P]-ATP-Mg revealed that the ^{32}P and HMGR enzyme activity were located in a single electrophoretic position. Dephosphorylation of ^{32}P-HMGR was associated with loss of Radioactivity and full restoration of enzyme activity. RK from human liver cytosol also exists in active and inactive forms. Active RK can be inactivated by incubation with phosphatase. Inactivated RK can be reactivated by incubation with ATP-Mg and a second kinase, reductase kinase kinase. These results represent the first demonstration of in vitro modulation of human liver HMGR by a bicycle cascade system involving reversible phosphorylation. We have also investigated the effect of cholesterol (CHL) and mevalonolactone (MVA) on the enzyme activity of rat liver HMGR. The effects of MVA and CHL are 2-fold. Short-term regulation involving inhibition of HMGR due to increase in phosphorylation; the long-term effect on HMGR is due to decrease in enzyme concentration.

PHS-6040
(Rev. 2-81)

Project Description

Objective:

1) *In Vitro* phosphorylation of Human liver HMGR.

Methods Employed:

(i) Preparation of Microsomes: 42 g of human liver was homogenized in 126 ml of 50 mM KCl, 40 mM KH_2PO_4, 30 mM EDTA (pH 7.2), 0.1 M sucrose, 5 mM DTT, 1mM sodium azide, (Buffer A), centrifuged at 10,000 x g for 15 minutes. The supernatant was then centrifuged at 105,000 x g for 90 minutes. The cytosol was carefully removed and used for analysis of reductase kinase and reductase kinase kinase. The microsomal pellet was suspended in 2 ml of a buffer containing 50 mM Tris-HCl, 50 mM KCl, 1 mM EDTA (pH 7.5), 0.25 M sucrose and 5 mM DTT, and stored frozed at -20°. 3g aliquots of livers were also homogenized in 9 ml of Buffer A containing either 50 mM or NaCl. The 10,000 x g, post mitochondrial supernatants from these two groups were utilized for isolation of microsomes by $CACL_2$ (8 mM) precipitation as previously described. The microsomes isolated by this rapid procedure were suspended in 2 ml of Buffer A containing either 50 mM NaCl or NaF, and stored frozed at -20°. Microsomal HMG-CoA reductase isolated by high speed centrifugation was solubilized and purified as described for the rat liver enzyme.

(ii) Isolation of Reductase Kinase: 20 ml of cytosol was fractionated with ammonium sulfate (0-30%). The precipatate was dissolved in 5 ml of 10 mM 2-(N-morpholine) ethane sulfonic acid (MES), pH 6.6 containing 5 mM DTT and 20% glycerol (Buffer C), desalted on Sephadex G-25M (PD10) columns and eluted with Buffer C containing 10 mM MgAC. The fraction was applied to 6 ml of agarose-hexane-ATP column, washed with 50 ml of 5 mM MES-10 mM MgAC (ph 6.6) containing 5 mM DTT and glycerol. The eluant containing reductase kinase activity was precipitated with ammonium sulfate at 35% saturation. The precipitate was dissolved in 1 ml of Buffer and stored frozed at -20°.

(iii) Analysis of Phosphorylated [^{32}P]-Labeled HMG-CoA Reductase: Purified HMF-CoA reductase was incubated for 30 min at 30° with 0.2 mM [^{32}P]-ATP (3 mCi), 5 mM MgAC in a medium containing 50 mM β-glycerophosphate (pH 7.0), 2 mM DTT and 130 mg of partially purified reductase kinase from human liver cytosol. The reaction was stopped by addition of EDTA, and an aliquot was used for HMG-CoA reductase activity. Control sample contained all components except HMG-CoA reductase. Following, phoslhorylation samples were dialyzed to remove salt and unbound radioactivity, concentrated by Amicon ultrafiltration (YM10 membrane) and used for polycrylamide gel electrophoresis under non-denaturing conditions. Samples were applied to a 7.5% acrylamide gel including a stacking gel as described by Maurer for gel system No. 6 except that the buffers contained 10 mM dithiothreitol. After electrophoresis, three gels were sliced into 3 mM lengths, one used for assaying HMG-CoA reductase activity, the second one for counting protein-bound radioactivity and the third for SDS-gel electrophoresis. Slices (3 and 4) containing HMG-CoA reductase activity and radioactivity were homogenized in 10 mM Tris-HCl (pH 8.0) containing 1% SDS, 40 mM dithiothreitol, 1 mM EDTA, 10 mg/ml pyronin y, incubated for 30 min at 70° and analyzed by SDS gel electrophoresis.

Major Findings:

The first evidence that human liver HMG-CoA reductase exist in inactive and active form was obtained from Ca^{++} precipitated microsomes isolated in the presence of NaF. The expressed enzymic activity of microsomal HMG-CoA reductase was lower when liver was homogenized in the presence of NaF in comparison to the control (NaCl) group. Incubation of microsomes with (protein phosphatase-1) fully restored the enzymic activity of microsomes from the NaF group. NaF apparently inhibited the phosphoprotein phosphatases during homogenization, thus preventing the activation (dephosphorylation) of inactive (phosphorylated) HMG-CoA reductase. The inactivation-reactivation of HMG-CoA reductase was confirmed in microsomes isolated by high speed centrifugation. The enzymic activity of HMG-CoA reductase was rapidly inhibited in the presence of ATP-Mg and excess of EDTA. The inhibition of HMG-CoA reductase was time, ATP concentration and temparature dependent. Incubation with ATP-Mg at 30° was associated with greater inactivation of HMG-CoA reductase than at 37°. Incubation of ATP-Mg inactivated enzyme with PP-1 resulted in a time dependent reactivation (dephosphorylation) of HMG-CoA reductase activity. The dephosphorylation reaction was completely blocked by NaF. To establish definitively that inactivation-reactivation of HMG-CoA reductase was due to phosphorylation-dephosphorylation, purified HMG-CoA reductase was incubated with partially purified reductase kinase and ($\gamma^{32}P$)-ATP-Mg at 30°. Thirty min of incubation (phosphorylation) caused 60% inactivation of HMG-CoA reductase activity. Analysis of the incubation mixture by polyacrylamide gel electrophoresis revealed that protein bound ^{32}P-radioactivity was present in the position corresponding to the enzymic activity of HMG-CoA reductase. Dephosphorylation of ^{32}P-labeled HMG-CoA reductase with PP-1 was associated with loss (90%) of radioactivity and increase in the enzymic activity. Incubation of HMG-CoA reductase without reductase kinase resulted in no ^{32}P incorporation.

Gel slices containing [^{32}P]-labeled HMG-CoA reductase and enzymic activity were homogenized in diluted (1:3) buffer A. An aliquot containing labeled gel electrophoresis, and radioactivity (>95%) was associated with the single electrophortic band of purified HMG-CoA reductase. The dephosphorylated samples revealed complete loss of HMG-CoA reductase bound radioactivity.

Objective:

2) In Vitro phosphorylation-dephosphorylation of human liver Reductase-Kinase (RK).

Methods Employed:

(i) Inactivation of Reductase Kinase: Microsomal or cytosolic reductase kinase was inactivated (dephosphorylated) by preincubation with phosphatase from rat liver. The incubation was terminated by the addition of 50 mM NaF.

(ii) Isolation of Reductase Kinase Kinase: Human liver cytosol was fractionated with ammonium sulfate. The protein precipitated at 65% saturation was dissolved in 5 mM Tris HCl, 1 mM EDTA, 50 mM NaCl (ph 7.5), 5 mM DTT, 10% glycerol, 50 mM phenylmethylsulfonyl fluoride (Buffer D), dialyzed overnight in the same buffer, applied to a column of phosphocellulose (1.6 x 6 cm), and eluted

with a gradient of 1 M KCL in buffer D (100 ml) and buffer D (100 ml). Fractions (4 ml) were collected and assayed for reductase kinase kinase activity. Fractions containing reductase kinase kinase activity (0.5 M to 0.7 M KCl) were pooled, dialyzed in buffer D, concentrated and stored frozen at -20°.

(iii) Assay of Reductase Kinase: Reductase kinase activity from microsomes, cytosol, or partially purified from cytosol was assayed in 50 mM β-glycerophophate (pH 7.0), 50 mM NaF, 2 mM DTT, 5 mM MgAC, varying amounts of ATP, 2 mg/ml BSA and appropriate samples of HMG-CoA reductase in a total volume of 100 or 200/ul. After incubation at 30° for varying lengths of time, 50 mM EDTA was added, followed by the reagents for HMG-CoA reductase assay containing 5 mM [^{3}H] mevalonolactone.

(iv) Assay of Reductase Kinase Kinase: Inactivated (dephosphorylated) reductase kinase samples were preincubated for 30 min at 37° with partially purified reductase kinase kinase, in a medium containing 50 mM MES buffer (pH 7.0) 2mM DTT, 2 mM ATP, 4 mM MgAC. Following preincubation, HMG-CoA reductase enzyme was added and incubation continued for an additional 30 min followed by HMG-CoA reductase assay.

Major Findings:

The active form of microsomal or cytosolic reductase kinase can be inactivated by incubation with PP-1. Inactivated (dephosphorylated) reductase kinase failed to inactivate HMG-CoA reductase in the presence of ATP-Mg. Incubation of inactivated reductase kinase with ATP-Mg and a partially purified cystosolic protein kinase, designated reductase kinase kinase, was associated with activation (phosphorylation) of reductase kinase and increase in the inactivation of HMG-CoA reductase. Reductase kinase kinase alone had no effect on the enzymic activity of HMG-CoA reductase. These results are interpreted as indicating that reductase kinase, like HMG-CoA reductase, exists as active and inactive forms. The active and inactive forms of the reductase kinase can be interconverted by a PP-1 and a protein kinase, reductase kinase kinase. The expressed reductase kinase activity always varied inversely to expressed HMG-CoA reductase activity.

Objective:

3) In Vivo Modulation of Rat Liver HMGR phosphorylation by cholesterol (CHL) and mevalonolactone (MVA).

Methods Employed:

Rats were maintained on a 3:00 AM to 3:00 PM dark cycle for 3 weeks prior to the study. Four rats in each group were injected with [^{32}P]-phosphoric acid (intraperitioneally, 2mCi at 7:30 AM and 8:30 AM). At 8:40 AM, saline (1 ml, control) or mevalonolactone (250 mg/ml saline) was administered by nasogastric tubes. At 9:00 AM, livers were removed, homogenized in 0.25 sucrose-50 mM Tris-HCl-10 mM EDTA-100 mM NaF-2 mM DTT (ph 7.2), microsomes isolated and HMG-CoA reductase purified. HMG-CoA reductase activity and its dephosphorylation were carried out as reported. For the cholesterol study, four rats in each group were administered either 2 ml of corn oil (control group) or 300 mg of cholesterol as

a corn oil suspension (cholesterol-fed group). 75 minutes after administration, livers were removed under anaesthesia, micromes isolated, and HMG-CoA reductase activity determined.

Major Findings:

Rats were injected with [^{32}P] phosphoric acid and administered 250 mg of either mevalonolactone or saline by nasogastric tube. Twenty minutes after mevalonolactone administration, there was a 36% inactivation of HMG-CoA reductase activity, and a significant increase in the incorporation of ^{32}P into purified HMG-CoA reductase. Enzymic activity of HMG-CoA reductase returned to control values following dephosphorylation with PP-1. Analysis of [^{32}P]-labeled HMG-CoA reductase by SDS gel electrophoresis revealed a single peak of radioactivity coincident with the electrophoretic position of purified HMG-CoA reductase. Treatment of [^{32}P] reductase with PP-1 was associated with loss of radioactivity (>95%) and restoration of enzymic activity. If microsomal HMG-CoA reductase was isolated and analyzed 60 minutes after administration of mevalonolactone, no evidence of increased inactivation (by phosphorylation) and reactivation by PP-1 was observed. However, 60 minutes after mevalonolactone feeding, HMG-CoA reductase activity was significantly inhibited (78%).

Seventy minutes after the feeding of cholesterol (1.2 g/kg, nasogastric tube) to rats, a 21% inactivation (phosphorylation) of HMG-CoA reductase was observed. Dephosphorylation of inactivated HMG-CoA reductase by PP-1 was associated with complete restoration of enzyme activity. HMG-CoA reductase isolated 2.5 hours after cholesterol feeding revealed no increase in phosphorylated enzyme or reactivation by PP-1.

Both mevalonolactone and cholesterol modulate the activity of HMG-CoA reductase by two mechanisms, the first mechanism involves short-term regulation by changes in the activity of the enzyme by phosphorylation. The long-term effect on HMG-CoA reductase is due to reduction in enzyme concentration. Thus, cholesterol and mevalonolactone modulate the activity of HMG-CoA reductase and hence cholesterol biosynthesis by both short-term (covelant modification) and long-term (syntheses and/or degradation) mechanisms. The mechanism by which these compounds increase the phosphorylation of HMG-CoA reductase is not yet understood. It is possible that under both situations, reductase kinase and/or phosphoprotein phosphatase are influenced in such a manner that conversion to the inactive or phosphorylated form of HMG-CoA reductase is favored.

The combined results reviewed in this report describe the precise short and long control of the enzymic activity of HMG-CoA reductase. Regulation of HMG-CoA reductase activity is of major importance in cellular metabolism since mevalonate serves as a precursor of for separate metabolic pathways including the biosynthesis of cholesterol, dolechols, ubiquinone, and isopentenyl tRNA.

Significance to Biomedical Research and the Program of the Institute

These results represent the first demonstration of *in vitro* modulation of enzymic activity of *human liver* HMG-CoA reductase by a bicylic cascade system in-

volving reversible phosphorylation of HMG-CoA reductase and reductase kinase. The existence of HMG-CoA reductase in interconvertible forms may provide a mechanism for short-term regulation of the pathway for cholesterol in man. These studies also suggest that the mechanism for short-term regulation of HMG-CoA reductase in rat and human liver is identical and the rat appears to be an excellent model for investigating the properties and regulation of HMG-CoA reductase. The elucidation of the mode of control of this enzyme in human and rat liver will allow a detailed analysis if the parameters involved in the cellular regulation of cholesterol metabolism in normal subjects and patients with atherosclerosis.

Proposed Course:

A systematic investigation of the modulation of HMG-CoA reductase in human and rat liver will be continued. The HMGR, reductase kinase, and reductase kinase kinase from human liver will be purified and characterized during the next year. In Vivo regulation of rat liver HMGR and RK under different physiological conditions (e.g. Dolichol, Ubiquinone, 7-ketocholesterol and 25-hydroxycholesterol administration) will also be investigated. These studies will enhance our understanding of the role of this pivotal enzyme in the cellular regulation of cholesterol in human and rat liver.

Publications:

1. Beg, Z. H., Stonik, J. A., and Brewer, H. B., Jr.: In Vitro and in vivo phosphorylation of rat liver 3-hydroxy-3-methylglutaryl coenzyme A reductase and its modulation by glucagon. J. Biol. Chem. 255: 8541-8545, 1980.

2. Beg, Z. H., Stonik, J. A., and Brewer, H. B.: Reversible phosphorylation of Heptic 3-hydrox-3-methylglutaryl coenzyme A reductase in Man, modulation of enzymic activity by a bicyclic cascade system. J. Biol. Chem., 1981, in press.

3. Beg, Z. H., and Brewer, H. B., Jr.: Regulation of Liver 3-hydroxy-3-methylglutaryl coenzyme A Reductase. Current Topics in Cellular Regulation. Volume 20, 1981, in press.

4. Beg, Z. H., and Brewer, H. B., Jr.: Modulation of Rat Liver 3-hydroxy-3-methylglutaryl coenzyme A reductase Activity be Reversible Phosphorylation. Federation Proceedings, 1981, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02017-03 MDB

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Theoretical Analysis of the Metabolism of Lipoproteins and Their Apolipoprotein and Triglyceride Moieties

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

PI:	Loren A. Zech, M.D.	Senior Investigator	MDB	NHLBI
Other:	Ernst J. Schaefer, M.D.	Senior Investigator	MDB	NHLBI
	H. Bryan Brewer, Jr., M.D.	Chief	MDB	NHLBI

COOPERATING UNITS (if any) Mones Berman, Ph.D., Laboratory of Theoretical Biology, NCI; Scott M. Grundy, M.D., Ph.D., University of Cal., La Jolla, CA; Barbara Howard, Ph.D., Peter H. Bennett, M.D., Phoenix Clinical Research, Center Section, NIAMDI ~~Waldo Fisher, M.D., Ph.D., University of Florida, Gainesville, FL.~~

LAB/BRANCH
Molecular Disease Branch

SECTION
Peptide Chemistry

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.4	PROFESSIONAL: 1.4	OTHER: 0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
Techniques were developed to examine the sensitivity of the ratio of the residence times of the two apoA-I components in the apoA-I model. Utilizing plasma, urine and whole body radioactivity data apoA-I and apoA-II compartmental models were constructed for both males and females. ApoA-II decayed by two pathways in both males and females. In males, 25% of apoA-II decayed through the minor pathway which took 22 hr. longer than the major decay pathway. In females, 30% was catabolized by the minor pathway with a delay of 31 hrs. ApoA-I has two distinct plasma compartments in both males and females. 28% of apoA-I is found in the second compartment for males, and 40% is present in females. In addition, while the second apoA-I component has the same residence time in males and females, the primary component in males has a residence time of 5.4 days and in females, 6.8 days. Triglyceride kinetics were studied in hyperlipidemic caucasian, and diabetic Pina Indians utilizing radiolabeled glycerol. Hypertriglyceridemia in subjects with genetically defined familial hyperlipidemia was associated with increased triglyceride production while in a group of patients with Type IV phenotype elevated triglycerides were secondary to decreased fractional catabolism.

PHS-6040
(Rev. 2-81)

Project Description

1) Objective:

Development of both compartmental and statistical models for analysis of apoA-I and apoA-II metabolism in normal subjects and patients with disorders of apolipoprotein metabolism, lipoprotein metabolism, atherosclerosis, and other lipid abnormalities.

Methods Employed:

The methods used for the development of multicompartmental models using turnover data from radiolabeled apolipoproteins and plasma lipoprotein studies have been detailed in previous reports. These models are stimulated using the SAAM simulator (a large collection of digital computer programs on the Univac 1108 computer and the National Bureau of Standards, the Peripheral Data Processor 10 at the National Institutes of Health, and the VAX/780 digital computer in the Laboratory of Theoretical Biology, National Cancer Institute). These simulated results are compared to the experimental results and the connectivity (number and topology of compartments) as well as flow of the model changed until a working model in developed. Using this model, the volume of distribution of the apolipoproteins is estimated and compared to independent estimates of these volumes. After development of the compartmental model, the parameters of the model are adjusted using nonlinear least square techniques resulting in a minimal lease square error. These have now been extended to studies in which differences in multicompartmental models, developed using turnover data from two separate radiolabels on two different apolipoproteins in normal individuals have been compared.

Major Findings:

1) Plasma, urine, and whole body data was separated into two groups based on sex resulting in average data on 9 females and 8 males. Plasma, urine and whole body kinetic data for apoA-I and apoA-II was compared and found to be different for both males and females.

2) In both males and females, apoA-II decayed through two separate pathways. While the second decay route accounts for 25% of the apoA-II decay in males, 30% is catabolized by this route in females. Compared to the primary or major path of catabolism, catabolism of apoA-II is delayed 22 hours within the secondary pathway for male subjects and 31 hours for female subjects.

3) In both males and females apoA-I has two distinct plasma components, a fast and slow decaying component. While the residence time of the fast component is equivalent when the metabolism in males and females is compared, 28% and 40% of the apoA-I respectively is catabolized through the fast component. In addition, the residence time of the slow component in males is 5.4 days while in females the slow component has a residency time of 6.8 days.

2) Objective:

Development of techniques for compartmental modeling which can be used in the analysis of lipid and lipoprotein metabolic studies in normal subjects and patients with disorders of apolipoprotein metabolism, lipoproteins metabolism, atherosclerosis, and other lipid abnormalities.

Methods Employed:

General methodology of compartmental modeling is extended and improved to provide the tools necessary to solve specific problems in the development of compartmental models for the analysis of lipid and lipoproteins kinetics. Recently, the concept of "sensitivity signal analysis" has been extended to compartmental models and fundamental relationships between various sensitivities determined.

Major Findings:

1) Sensitivity analysis in compartmental modeling applied to the previously developed apoA-I compartmental model has determined that the kinetics of apoA-I are very sensitive to changes in the quantity and residence time of apoA-I which is catabolized by a second plasma component, Fig. 1.

2) Several identities have been determined between sensitivities for compartmental models. If the sensitivity of p with respect to x and q with respect to x are designated S_x^p and S_x^q, when p, q are functions of x and the sensitivity of k with respect to x is designated $S_x^k = 0$ when k is not a function of x, then the following identities hold:

†SENSITIVITY S_ϱ^μ

$\varrho \backslash \mu$	y	$1/y$	ky	zy	y/z	y^k	e^y
x	S_x^y	$-S_x^y$	S_x^y	$S_x^z + S_x^y$	$S_x^y - S_x^z$	kS_x^y	yS_x^y
$1/x$	$-S_x^y$	S_x^y	$-S_x^y$	$-S_x^z - S_x^y$	$S_x^z - S_x^y$	$-kS_x^y$	$-yS_x^y$
kx	S_x^y	$-S_x^y$	S_x^y	$S_x^z + S_x^y$	$S_x^y - S_x^z$	kS_x^y	yS_x^y
zx	$\frac{1}{S_x^y + S_x^z}$	$\frac{-1}{S_x^y + S_x^z}$	$\frac{1}{S_x^y + S_x^z}$	$\frac{1}{S_x^y + zS_x^z}$	$\frac{1}{S_x^y}$	$\frac{1}{kS_x^y + S_x^z}$	$\frac{1}{yS_y^x + S}$

† $y, z = f(x)$ and $k \neq f(x)$

3) Objective:

1) Continued development of a general multicompartmental model for plasma lipoprotein metabolism through the examination of apoB metabolism.

2) Comparison of the parameters describing apoB metabolism when the apoB is labeled by an *in vivo* technique with those previously determined by *in vitro* labeling.

Methods Employed:

A group of subjects with polydispense LDL were given radiolabeled leucine, a precursor amino acid for protein biosynthesis. Plasma leucine, VLDL, LDL specific activity were determined following the injection of radiolabeled leucine. This data was used to construct a compartmental model. The kinetic parameters calculated from the *in vivo* compartmental model were compared with previous results from studies employing *in vitro* apoB labeling.

Major Findings:

1) A precursor-product relationship exists between VLDL and intermediate density lipoproteins (IDL), low density (S_f 10) lipoproteins, and finally low density (S_f 4) lipoproteins.

2) Lipoproteins were secreted directly into VLDL and IDL, and catabolized from both IDL and LDL.

3) The synthesis and fractional catabolic rates were comparable to those previously derived using lipoproteins labeled in vitro further verifying the general model for VLDL and LDL metabolism.

4) Objective:

1) Continued development of a multicompartmental model for triglyceride metabolism and the precursors of plasma lipoprotein triglycerides.

2) Estimation of the parameters describing triglyceride metabolism including synthesis rate, residence time, and fractional catabolic rate in groups of subjects with genetically determined hyperlipidemia.

Methods Employed:

1) Cohorts of subjects were selected by pedigree analysis and designated as having primary hypertriglyceridemia, familial combined hyperlipidemia, or non-monogenic hypertriglyceridemia.

2) Radiolabeled glycerol was administered to patients in each of the three groups. The topological structure (connectivity and numbers of compartments) of multicompartmental models previously developed for triglyceride metabolism were examined for their ability to explain the experimental results in each group. After it was established that the current model could explain the observed kinetic data, the kinetic parameters of VLDL-triglyceride metabolism were calculated for each group.

Major Findings:

Analysis of kinetics of VLDL triglyceride kinetics was performed in collaboration with Dr. Mones Berman (Laboratory of Theoretical Biology, NCI), and Dr. Scott Grundy (Veterans Administration Hospital, La Jolla, CA.) In non-monogenic patients with hypertriglyceridemia the transport rates of VLDL-triglyceride (mg/hr) were not increased and the hypertriglyceridemia was secondary to a decreased fractional catabolic rate (FCR). In the familial combined hyperlipidemic group hypertriglyceridemia was associated with increased transport (production) rate however, a small subgroup of subjects also had a decreased fractional catabolic rate. While synthesis rates for cholesterol and bile acids were variable in patients with familial combined hyperlipidemia (as measured by balance techniques), overproduction of sterols is characteristic of this disorder. In contrast, most subjects in the non-monogenic hypertriglyceridemia group did not have increased sterol synthesis. Data on the third group with familial hypertriglyceridemia is currently being analyzed and additional patients are being recruited.

5) Objective:

An analysis of the VLDL triglyceride kinetics of the Pina Indians.

Methods Employed:

The Pima Indian nation of Native Americans are an interesting, well-defined group of subjects. Studies of this group were performed in collaboration with Dr. Barbara Howard and Dr. Peter Bennett (Phoenix Experimental Station, NIAMDD). These individuals are of interest because they have an increased incidence of diabetes, decreased incidence of obesity, and greater than 150% of ideal body weight. In addition, they also represent a well-defined group in which insulin, glucose, cholesterol, and LDL triglyceride kinetics have been previously studied.

VLDL-triglyceride was endogenously labeled following the injection of 300μCi of the radiolabeled precursor, ^{3}H-glycerol. In addition, plasma levels of insulin, glucose and free fatty acids were determined by standard techniques detailed in previous annual reports.

The kinetics of VLDL-triglyceride metabolism in normal triglyceridemia, non-diabetic, male, Pima Indians has been established and the relationships among the kinetic parameters (fractional catabolic rate, synthesis rate, delipidation rate, etc.) and plasma values of glucose, insulin and free fatty acids have been examined in detail. A group of 10 age matched male normal triglyceridemic diabetics have been examined using the compartmental model.

Major Findings:

1) The connectivity (number of compartments, and topology of tracer flow) of the model developed for the diabetic Pima Indians is equivalent to the model previously developed for both non-diabetic Pima Indians and caucasian males.

2) Studies have been completed in 10 diabetic Pima Indians and the data is currently under analysis. Additional studies are currently underway to provide a sufficient number of patients for statistical comparison with data previously collected on non-diabetic Pima Indians and caucasians.

Significance to Biomedical Research and the Program of the Institute:

Understanding the metabolism of lipoproteins and the moieties which make up these lipoproteins (cholesterol, cholesterol ester, triglycerides, and apolipoproteins) are significant because of their relationship to atherosclerosis and cholelithiasis. The development of a concept of plasma lipoprotein metabolism is approached by the theoretical analysis of data from metabolic studies using the techniques of compartmental and statistical model building. This type of theoretical analysis provides a framework for discussion between investigators. This project consists of the testing and further development of compartmental models for lipoprotein metabolism as well as the proposal of new models where they do not exist.

Understanding lipoprotein metabolism is of major importance due to the central role of lipoproteins in the transport and catabolism of cholesterol and triglycerides in normal and patients with disorders of lipid metabolism and/or atherosclerosis. Because of the recent elucidation of the negative correlations between HDL-cholesterol levels and the incidence of coronary heart disease the understanding of the two major HDL apolipoproteins (apoA-I and apoA-II) is particularly relevant to the understanding of atherosclerosis.

Understanding the modulation of lipoprotein, cholesterol, cholesterol ester, triglycerides and apolipoproteins by drugs, diet, and genetic disease is also of significance since changes in these effectors may have major effects on atherosclerosis and cholelithasis. This theoretical analysis also provides a framework for comparison between groups as dissimilar as caucasians and American Indians.

Proposed Course:

Detailed studies will be continued on the analysis of the differences in apoA-I and apoA-II metabolism by further specifying and defining the current compartmental models, with particular emphasis on analysis of studies in abnormal subjects such as type I and Tangier subjects. Additional diabetic Pima Indians will be examined to increase the sample size so that valid comparisons between groups can be performed. Preliminary studies will be initiated on the compartmental analysis of apoE and apoC metabolism. The overall objective will be the development of a comprehensive model of human lipoprotein metabolism by the incorporation of this formation into previously proposed LpB models. The formulation of an overall conceptualization of lipoprotein metabolism will be continued by qualitative and quantitative testing of these conceptions using compartmental modeling and other theoretical methods. Of particular interest will also be the determination of which parameters are modified by diet, drug, and transformed by genetic disease.

In addition, laboratory examination of type V subjects using the cholesterol balance method and the triglyceride, radiolabeled glycerol method will be carried out on 4 to 8 inpatients.

Also, the concepts of sensitivity analysis applied to compartmental analysis will be further developed and expanded to aid in detailed studies proposed above.

Publications:

1) Zech, L. A., 1980, Sensitivity in Compartmental Models. In models of Lipoprotein Kinetics, Proceedings of the 1979 Phoenix Meeting. Edited by Mones Berman. Academic Press. (in press).

2) Fisher, W. R., L. A. Zech, P. Bandalaye, G. Warmke, and M. Berman. 1980, The metabolism of apolipoprotein B in subjects with hypergriglyceridemia and polydispense LDL. J. Lipid Res. 21: 760-774.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02019-03 MDB

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Metabolism of Human Plasma Apolipoproteins

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

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LAB/BRANCH

Molecular Disease Branch

SECTION

Peptide Chemistry

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 2	PROFESSIONAL: 1	OTHER: 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)

Apolipoprotein E (apoE) plays a central role in the metabolism of remnants of triglyceride (TG)-rich particles. ApoE can be separated into 3 major bands (E_2, E_3, and E_4) with each band being inherited in a condominant fashion, the 3 bands being coded by 3 separate alleles at one genetic locus. ApoE_2 is the only major band found in most subjects with Type III hyperlipoproteinemia (HLP). In the past year studies have been performed to compare the metabolism of ApoE from a subject homozygous for E_2 with apoE from a subject homozygous for E_4. In addition, a sensitive double antibody radioimmunoassay for apoE has been developed.

ApoE_2 was found to have a residence time twice as long as E_4 in both normal and Type III HLP subjects and the elevated apoE level found in Type III subjects was shown to be solely due to a catabolic defect of their apoE_2. Rat hepatocytes were found to bind apoE_4 more avidly than apoE_2. These findings are consistent with the hypothesis that the hyperlipidemia found in Type III HLP subjects is due to an abnormal apoE (apoE_2) which causes a defective catabolism of remnants of TG-rich particles by the liver.

PHS-6040
(Rev. 2-81)

Objective:

Our objective was to study the metabolism of apoE. Specifically, studies were designed to determine the *in vivo* kinetic parameters of the major polymorphic forms of apoE in humans. In addition, studies were undertaken to investigate the interaction of purified apoE isoforms with hepatocytes. The studies should provide information into the metabolism of remnants of TG-rich particles, further insights into their atherogenic potential, and a better understanding of the pathophysiology of Type III HLP. One has to have a sensitive and specific assay for apoE to perform these studies. Therefore, we also wanted to develop a double antibody radioimmunoassay for apoE.

Methods:

ApoE was isolated to homogeneity from individuals homozygous for $ApoE_2$ and $apoE_4$ using ultracentrifugation, heparin affinity chromatography, and gel filtration chromatography.

Isolated apoE was used to raise antibodies in rabbits by standard immunization methods. ApoE was iodinated by the iodine monochloride method and a sensitive double antibody radioimmunoassay has been developed.

$ApoE_2$ and $apoE_4$ were iodinated by the iodine monochloride method and injected into normal and Type III HLP subjects. Plasma was obtained at various times and separated into lipoprotein density fractions by ultracentrifugation.

The rate of decay of radioactivity from these fractions was determined. Plasma residence times were determined using a computer modeling program (SAAM 27), and plasma fractional catabolic rates and production rates were calculated.

Radioiodinated $apoE_2$ and $apoE_4$ were incubated with isolated rat hepatocytes, endothelial cells, and Kupffer cells. The rate and amount of binding and internalization of apoE were determined.

Major Findings:

1) ApoE can be measured in our radioimmunoassay at a concentration as little as 0.1 mg/dl. Plasma apoE levels in normal individuals are approximately 2-5 mg/dl.

2) Compared to $apoE_4$, $apoE_2$ has abnormal binding characteristics to plasma lipoproteins. $ApoE_2$ is present more in HDL and in plasma proteins unassociated with lipoproteins, and less in VLDL.

3) $ApoE_4$ is catabolized very rapidly in normal subjects and approximately twice as fast as in Type III HLP subjects.

4) $ApoE_2$ is catabolized approximately one-half as fast as $apoE_4$ in both normal and Type III HLP subjects. This difference is found also in all of the lipoprotein fractions in both types of subjects.

5) The production rate of apoE is the same in Type III HLP subjects as in normal individuals. Therefore the elevated apoE levels found in Type III HLP subjects is due to decreased catabolism and not to oversynthesis.

6) Rat hepatocytes, endothelial cells, and Kupffer cells bind and internalize $apoE_4$ more rapidly and to a greater extent than $apoE_2$. Of these three cell types, hepatocytes are more active than the other cell types in this process.

7) All of these findings are consistent with the hypothesis that apoE is important in the catabolism of remnants of TG-rich particles by the liver and Type III HLP is associated with an abnormal apoE which causes a catabolic block of these remnant particles.

Significance to Biomedical Research and the Program of the Institute:

Remnants of TG-rich particles are atherogenic in both animal models and humans and apoE plays a central role in the catabolism of these particles. In addition, an abnormal apoE is associated with Type III HLP, a disease characterized by the accumulation of TG particle remnants. Our studies have given further insights into the role of apoE in the metabolism of TG particle remnants by the liver and have clearly demonstrated that the different apoE isoforms have different rates of metabolism. We have shown that $apoE_2$ from Type III HLP subjects is catabolized slower than $apoE_4$ and this leads to an accumulation of remnants in these individuals. Through these studies we are gaining knowledge concerning the control of lipoprotein metabolism by apoE and about the basic metabolic defect in one of the diseases associated with premature atherosclerosis in humans.

Proposed Course:

It is planned to continue studying apoE metabolism from a number of different perspectives. Additional *in vivo* human studies will be performed to compare $apoE_3$ with $apoE_2$ and $apoE_4$. Type III subjects will also be studied on and off therapeutic drug regimens. In addition, apoE interactions with human isolated cell membranes and cell tissue cultures will be studied to gain a better understanding of the metabolism of apoE isoforms at a cellular level.

Publications:

R. E. Gregg, L. A. Zech, E. J. Schaefer, and H. B. Brewer, Jr.: Type III Hyperlipoproteinemia: Defective Metabolism of an Abnormal Apolipoprotein E. *Science* 211: 584-586, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02021-01 MDB

PERIOD COVERED
October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)

Sterol Metabolism in Patients with Betasitosterolemia

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

PI:	Richard E. Gregg, M.D.	Clinical Associate	MDB	NHLBI
Other:	H. Bryan Brewer, Jr., M.D.	Chief	MDB	NHLBI

COOPERATING UNITS (if any)
William Connor, M.D., Dept. of Medicine, University of Oregon Health Center Portland, Oregon.

LAB/BRANCH
Molecular Disease Branch

SECTION
Peptide Chemistry

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 0.2	PROFESSIONAL: 0.2	OTHER: 0

CHECK APPROPRIATE BOX(ES)
☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Betasitosterolemic patients have xanthomas in childhood, markedly premature atherosclerotic cardiovascular disease, and elevated levels of plant sterols, primarily betasitosterol. We have shown that in addition to over absorbing dietary plant sterols, they also over absorb shellfish sterols. This suggests that betasitosterolemic patients have a generalized overabsorption of dietary sterols. The molecular defect in this disease appears to be due to an abnormality at the site for discrimination for sterol absorption. In normal individuals plant and shellfish sterols are not absorbed whereas cholesterol is actively transported into the body. In patients with betasitosterolemia there is no selectivity to sterol absorption, and cholesterol, plant sterols and shellfish sterols are absorbed in significant quantities.

PHS-6040
(Rev. 2-81)

Objectives:

It has previously been shown that betasitosterolemic patients have an increased absorption of dietary betasitosterol and their absorption of cholesterol is in the high normal range. The object of this study was to determine if the over absorption of sterols was limited to plant sterols or if it was a pansterol over absorption abnormality.

Methods Employed:

Shellfish (scallops, oysters, and clams) contain significant quantities of a number of unusual sterols in addition to cholesterol. A patient with betasitosterolemia was placed on a low fat, low cholesterol, low plant sterol, and a no shellfish sterol baseline diet. She was then given a diet containing 500 gm of shellfish per day. Her plasma levels of the different shellfish and plant sterols were determined before and after the diet, the stool was collected to determine sterol absorption by the sterol balance method and bile samples were collected before and after the diet to determine the relative biliary excretion of these sterols. Control values in normal subjects have previously been determined by Dr. William Connors (Dept. of Medicine, Univ. of Oregon Health Center, Portland, Oregon).

Major Findings:

1) Plasma levels of the shellfish sterols were markedly elevated in the betasitosterolemic patient compared to the normal controls.

2) The betasitosterolemic patient over absorbed all of the shellfish sterols.

3) The betasitosterolemic patient excreted the shellfish sterols in the bile.

Significance to Biomedical Research and the Program of the Institute:

Betasitosterolemic patients, now more appropriately called hypersterolemic patients, have an over absorption of all sterols tested so far. In addition, published reports have shown premature cardiovascular disease and xanthomas in adolescence with normal to mildly elevated plasma sterol levels and a relatively decreased excretion of plasma betasitosterol in bile. This suggests that their abnormality affects sterol metabolism at a number of different sites, and when the defect is elucidated at a molecular level it should give basic insights into the recognition and regulation of sterol absorption, sterol accumulation in xanthomas, and sterol excretion in bile.

Proposed Course:

It is planned to study sterol metabolism in this patient while she is on different therapeutic drug regimens. In addition her fibroblasts are in tissue culture and these will be used to study the defect in this disease at a cellular and molecular level.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02022-01 MDB

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Cellular Lipid and Lipoprotein Biochemistry and Metabolism

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

P.I.:	Jeffrey M. Hoeg, M.D.	Research Associate	MDB	NHLBI
Others:	H. Bryan Brewer, Jr., M.D.	Chief	MDB	NHLBI
	Stephan Demosky	Chemist	MDB	NHLBI
	Briston Williamson	Laboratory Technician	MDB	NHLBI

COOPERATING UNITS (if any)
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Division of Pediatric Gastroenteronology
University of Utah, Salt Lake City, Utah

LAB/BRANCH
Molecular Disease Branch

SECTION
Peptide Chemistry

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland

TOTAL MANYEARS: 3.2	PROFESSIONAL: 2.2	OTHER: 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 in our laboratory is directed toward the study of lipoprotein-cell interactions as well as the intracellular control of cholesterol, cholesteryl ester, and triacylglycerol metabolism in human skin fibroblasts. Over the past 12 months, we have developed assays for human fibroblast acid ester hydrolase and neutral ester hydrolase. We have used these assays to characterize cholesteryl ester and triglyceride catabolism in normal cells as well as in cells from a new case of Wolman's Disease, cholesteryl ester storage disease and a previously uncharacterized lipid storage disease. We have also devised a new technique to measure lipoprotein binding to fibroblasts and are evaluating these interactions in cells from normal as well as patients with dyslipidemia and atherosclerosis. Studies addressing substrate specificity for sterol-ester metabolism in fobroblasts from normal humans and from patients with beta-sitosterolemia have been completed. Finally, outpatient studies evaluating Neomycin and Niacin in type II hyperlipoproteinemia and Niacin in types III and V hyperlipoproteinemia have been designed and instituted.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives:

1) To develop assays for the enzymes catabolizing intracellular ester bonds.

2) To devise a new method characterizing lipoprotein-cell interactions in cell lines growing in tissue culture.

3) To evaluate and define substrate specificity in sterol-ester, and glyceryl-ester catalysis.

Methods Employed:

As outlined in our objectives, our primary aim this year was to develop methods for study of the lipid metabolism of human fibroblasts. Thus, our methods are more clearly outlined under major findings.

Major Findings:

1) Enzymes that hydrolyze cholesteryl ester and acylglycerol bonds have been previously described for enzymes in rat liver. In order to study the control of the enzymes regulating the ester pools in human fibroblasts, we have established reproducible assays for the enzymes that catabolize these lipid ester binds. Methods for the quantitation of neutral ester hydrolase and acid ester hydrolase were developed utilizing radiolabeled and fluorence substrates.

2) Wolman's disease is characterized by steatorrhea, hepatosplenomegaly, absence of hepatic acid lipase, and death in infants. Using new sensitive methods, we have quantitated both the acid ester hydrolase (AEH) and neutral ester hydrolase (NEH) for the first time in nonhepatic tissue. Our finding that AEH is absent but NEH persists in a patient with Wolman's Disease are consistent with the view that these two enzymes are independently synthesized.

3) Previous studies in a variety of laboratories have attempted to measure low density lipoprotein (LDL) binding to adherent cell lines growing in tissue culture. Because fibroblasts are adherent to their culture vessels, measurement of binding kinetics has been difficult. We have developed a new microculture technique for growing fibroblasts attached to beads. Using this technique, we have developed an assay method which can now address reversible ligand-receptor interactions which have not previously been possible. This method will be of major importance in delineating the binding affinities, and number of receptor sites involved in lipoprotein-cell interactions.

4) Patients with beta-sitosterolemia absorb plant and shellfish sterols not generally utilized by normal man. These sterols and steryl esters are present in the blood and tissues of these patients. We studied the ability of normal fibroblasts and fibroblasts from a patient with beta-sitosterolemia do hydrolyze cholesterol and beta-sitosteroyl ester, and to esterify the free sterol. These sterols were incorporated into LDL and incubated with fibroblasts esterified both

steryl ester bonds. Therefore, beta-sitosterolemia does not appear to be due to a loss of the ability of intracellular enzymes to distinguish different sterols or steryl esters.

Significance of Biomedical Research and Program of the Institute:

These methods can now be used to assess sterol and ester metabolism in hepatic and nonhepatic tissue in normal subjects and in patients with disorders of sterol metabolism. In addition, possible causal relationships with atherogenesis can now be evaluated.

Proposed Course:

The new techniques and methods described in this report will be used to evaluate normal tissues as well as tissues from patients with dyslipoproteinemia at the Clinical Center. Normal as well as abnormal lipid and lipoprotein metabolism can now be assessed and studies along these lines are underway. Ongoing outpatient drug studies evaluating the drug and diet treatment of hyperlipidemia will be continued.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02023-01 MDB

PERIOD COVERED October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Definition of the biochemical and physiological characteristics of hepatic lipase (HL) and lipoprotein abnormalities in primary biliary cirrhosis

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

P.I.:	Claus E. Jahn, M.D.	Guest Worker	MDB	NHLBI
	H. Bryan Brewer, Jr., M.D.	Chief	MDB	NHLBI
	Ernst J. Schaefer, M.D.	Senior Investigator	MDB	NHLBI
	James Osborne, Ph.D.	Senior Investigator	MDB	NHLBI
	Grace Huff	Chemist	MDB	NHLBI
	Lila Taam	Chemist	MDB	NHLBI
	Elizabeth Rubalcaba	Chemist	MDB	NHLBI
	Leslie Jenkins	Biologist	MDB	MHLBI

COOPERATING UNITS (if any)

Anthony Jones, M.D., NIADD
Michael Pawlita, M.D., NCI

LAB/BRANCH

Molecular Disease Branch

SECTION

Peptide Chemistry

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.8	PROFESSIONAL: 1.4	OTHER: .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)

Human post heparin plasma contains lipoprotein lipase (LPL) and hepatic lipase (HL), two major enzymes involved in lipoprotein metabolism. Studies were undertaken to further delineate the role of HL in lipid metabolism. A plasma activator of HL was isolated, and shown to be apolipoprotein A-II. A major substrate for HL is HDL, and HL appears to play a major role in the catabolism of the lipid component of HDL_2. The cofactor function of apoA-II for HL, is analagous to the cofactor function of apoC-II for LPL. Studies of patients with primary biliary cirrhosis revealed two groups of patients. One group had decreased HL activity, high HDL, and increased apoA-I and apoA-II; the second group had decreased HDL, and an increase in a new lipoprotein, LpX. Studies have also been initiated on the purification of HL, and the production of monoclonal antibodies to HL.

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

1) Determination of the plasma component activating the enzymic activity of HL.

2) Purification of HL from human post heparin plasma.

3) Characterization of HL activity in disease states.

4) Characterization of the plasma lipoproteins in chronic liver disease.

Methods Employed:

Methods for isolation and quantitation of plasma lipoproteins and apolipoproteins have been previously described as well as the methodology for production of monoclonal antibodies in last years annual report.

Major Findings:

1) Serum stimulates HL enzymic activity 2-3 fold. Initial studies in our laboratory confirmed the activation by using plasma. Subfractionating plasma into its lipoprotein components VLDL, LDL, HDL and the plasma proteins showed that VLDL inhibited HL by 25%, LDL had no effect on HL and HDL activated HL by 365%. Further fractionation of HDL into its lipid and apolipoprotein components identified apolipoprotein A-II as the activating plasma component for HL enzymic activity. ApoA-II activated HL by 392%. All other HDL apoproteins, apoA-I, apoC-I, apoC-II, and apoC-III, assayed inhibited HL.

2) Isolation of HL has been difficult due to the fact that the enzyme is very unstable during purification. We have undertaken the preparation of a specific antibody against HL by using the monoclonal antibody technique. It was possible to raise an HL antibody in Balb C mice and to obtain hybridomas. Specific hybridoma has been cloned and are being screened. The eventual development of a monoclonal antibody to HL will markedly facilitate the isolation of HL.

3) The physiological role of HL is incompletely understood, however evidence from our studies indicate that HL might be important in the metabolism of HDL particles. It was therefore of interest to study activity in patients with primary biliary cirrhosis. There have been reports indicating decreased HL activity in patients with liver disease. We found decreased HL activity in the majority of our patients and related these changes in enzymic activity to alterations in plasma lipoprotein and apoproteins. These studies provided additional indirect evidence for the role of HL in HDL metabolism; the activity of HL was correlated with HDL cholesterol ($r=-.8$, $p=.01$) as well as a phospholipid ($r=-.8$, $p=.01$).

4) The plasma lipoproteins have been characterized in 11 patients with primary biliary cirrhosis. Two distinct lipoprotein patterns were observed in this disease. In one group the major abnormalty was an increase of lipoprotein particles within HDL characterized by a high content of apoE; in the second group there was an additional increase in a new lipoprotein designated LP-X. Both

groups were also characterized by significant changes in plasma apolipoprotein concentrations for apoA-I, apoA-II, apoB and apoC-II indicating that profound disturbances of apolipoprotein metabolism occur in this disease.

Patients with primary biliary cirrhosis therefore have decreased HL and LPL activity and significant alterations in plasma apolipoprotein and lipoproteins. The studies provide new insights into the metabolic interrelationships between plasma lipoproteins and the HL and LPL lipolytic enzymes.

Significance to Biomedical Research and the Program of the Institute

The role of HL has been defined as well as the role of apoA-II in lipoprotein metabolism. ApoA-II has been identified during the course of these studies as an *in vitro* activator of HL which is very similar to the activation of apoC-II and LPL. This observation also suggests, like studies from other laboratories, that HL acts primarily in the metabolism of HDL. In addition, these results combined with the data evolving from the study of a group of patients with primary biliary cirrhosis provide insight into the pathophysiology of changes in plasma lipoproteins in obstructive liver disease.

Proposed Course:

Studies will be continued on the metabolic role of HL and apoA-II in lipoprotein metabolism, as well as the characterization of changes in apo- and lipoprotein metabolism in diseases secondary to HL deficiency. In addition the isolation of HL and production of a monoclonal antibody to HL will be continued. The ultimate purification of HL will provide the opportunity for a detailed analysis of the structural and function of this important enzyme in lipoprotein metabolism.

Publications:

Jahn, C. E., Osborne, J. C., Jr., Schaefer, E. J., and Brewer, H. B., Jr.,: *In vitro* activation of the enzymic activity of hepatic lipase by apoA-II. FEBS Letters (in press).

Annual Report of the
Laboratory of Molecular Hematology
National Heart, Lung, and Blood Institute
October 1, 1980 to September 30, 1981

The Laboratory of Molecular Hematology (LMH) studies the basic molecular mechanisms of gene expression and protein synthesis, specifically using hemoglobin as a model system. LMH is closely associated with the Clinical Hematology Branch (CHB) and collaborates on a number of joint projects. LMH is composed of three segments: the Section of Molecular Genetics, which is primarily concerned with the molecular control of eukaryotic gene expression; the Section of Molecular Cloning, which is primarily concerned with the isolation and characterization of globin and other genes from the genomes of eukaryotic cells; and the Section of Protein Biosynthesis, which is primarily concerned with the mechanism and regulation of hemoglobin synthesis at the translational level.

SECTION OF MOLECULAR GENETICS AND SECTION OF MOLECULAR CLONING

The immediate objectives of these Sections are to: (1) identify, isolate and characterize the regulatory factors of animal and human DNA which are involved in the control of the expression of the globin genes; (2) analyze the genomic DNA sequences involved in the regulation of gene expression in humans and animals; and (3) develop methods for transferring functional genes into tissue culture cells and intact animals. Information from these programs will be used to study the regulation of globin gene expression in normal and in thalassemic DNA. The long-term goal is to develop means whereby globin gene expression can be corrected in patients with β-thalassemia and other diseases involving abnormal hemoglobin biosynthesis.

Regulatory factors controlling the expression of the globin genes have been identified by a combination of cell biology and molecular biology techniques. Somatic cell hybrids, obtained by fusion of human or animal cells with mouse erythroleukemia (MEL) cells, have been used to provide evidence for positive and negative regulatory factors controlling globin gene expression. In order to purify and characterize these putative regulatory factors, intact-cell and cell-free assays have been established.

Besides somatic cell hybridization, several other methods of gene transfer are being used to insert genes into tissue culture cells and mouse embryos. The most successful of these has been the technique of physical microinjection in which a single copy of a specific gene can be injected into the nucleus of a single cell under conditions whereby the injected cell (or fertilized egg) can be grown in culture (or in a surrogate mother) into a cloned population (or a living animal). This technique was successfully used to correct a mouse thymidine kinase (TK) negative L cell by microinjection of a bacterial plasmid containing a functional TK gene.

The Sections have succeeded, during the past year, in demonstrating that: (1) A genetically defective tissue culture cell (TK^- mouse L cell) can be corrected not only by microinjection of the normal TK gene as part of a recombinant plasmid, but also by injection of a purified DNA fragment containing only the TK gene.

(2) The physical structure of the microinjected genes which are being replicated

in the genetically corrected L cells is a long head-to-tail tandem repeat (called a concatamer). Some of these concatamers (which appear to be greater than 100 kb in size) may be integrated into the mouse genome.

(3) DNA synthesizing techniques can be used to make specific gene probes. A probe has been synthesized that is an 18 base pair complement to a region between the 3' coding end and the poly A recognition site of human β-globin mRNA. This probe should allow the isolation of normal (or abnormal) human β-globin mRNA from a cell producing a number of different globin mRNAs.

(4) A cell-free transcription assay, originally described by Roeder and his colleagues, can be used in screening MEL cell fractions for transcriptional initiation factors. A crude extract of MEL cells has been separated into a number of active fractions, one of which, Fraction A, is now being purified to homogeneity.

(5) The three known strains of α-thalassemic mice are true models of the human α-thalassemias. The compensatory mechanism for partly alleviating the effects of the gene deletion in these mutated mice has been shown to exist in another mouse mutant which contains a duplicated β-globin locus.

SECTION OF PROTEIN BIOSYNTHESIS

Regulation of gene expression at the level of messenger RNA translation is an important aspect of interferon-mediated antiviral activity, cell growth, and differentiation and the overall coordination of cell metabolism. Translational regulation is frequently mediated by the covalent modification of existing protein factors required for protein synthesis. It is the objective of this Section to: (1) determine and characterize the molecular basis of translational regulation; (2) identify and characterize protein and nucleic acid components participating in these regulatory mechanisms; and (3) examine the regulation of globin gene expression in normal and disease (e.g., thalassemic) states.

During the past year, the Section has successfully:
(1) Demonstrated that both phosphorylation/dephosphorylation and oxidation/reduction modifications of eIF-2 can regulate protein synthesis initiation.

(2) Developed high performance liquid chromatographic procedures for the rapid isolation of eIF-2 phosphopeptides.

(3) Quantitated the extent of exogenous and endogenous eIF-2 phosphorylation in the reticulocyte lysate.

(4) Demonstrated ADP-ribosylation of the presumptive GTP-binding regulatory subunit of eIF-2.

(5) Purified and characterized a new protein complex required for catalytic recycling of eIF-2.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02212-05 MH

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Molecular Control of Eukaryotic Gene Expression

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

PI:	W. F. Anderson	Chief	MH NHLBI
OTHER:	B. Safer	Medical Officer	MH NHLBI
	E. Tolunay	Visiting Fellow	MH NHLBI
	Y. Chiang	Chemist	MH NHLBI
	W. Kemper	Chemist	MH NHLBI
	K. Li	Biologist	MH NHLBI
	L. Yang	Biologist	MH NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Molecular Hematology

SECTION
Section of Molecular Genetics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 4.9	PROFESSIONAL: 1.2	OTHER: 3.7

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)

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 has been obtained for the existence of a positive regulatory factor(s) in induced 2S mouse erythroleukemia cells. Attempts are being made to isolate, purify and characterize regulatory factors using a cell-free transcription system.

PHS-6040
(Rev. 2-81)

Objectives: The objective of this project is to isolate the factors involved in the expression of eukaryotic genes (specifically, those for hemoglobin) and to understand how they interact with the regulatory sequences of DNA in order to control gene expression within the intact cell. We have already shown that mouse erythroleukemia (MEL) cells, which are used as a model system for red blood cell differentiation, contain a positive regulatory factor involved in globin gene expression. The procedure is to fractionate these cells into various components which can be individually tested either by physical microinjection into target cells or in cell-free transcription assays for their ability to alter globin gene expression.

Methods:

1) Cells are grown under standard tissue culture conditions. MEL cells are fused by means of inactivated Sendai virus (or polyethylene glycol) to other somatic cells which normally are either capable or incapable of synthesizing hemoglobin. Chromosome and isozyme analyses are done by standard cell biology techniques.

2) Cell hybrids are analyzed for their ability to synthesize globin messenger RNA (mRNA) or globin. Globin mRNA is detected 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.

3) The cell-free transcription assay described by Roeder and his colleagues has been modified for use with MEL cell extracts. Standard protein fractionation techniques are used to isolate individually-active MEL cell fractions.

Major Findings:

1) Somatic cell hybrids (M11X-2, M11X-3) selectively retain human chromosome (HC) 11. These MEL hybrids were obtained by fusing HGPRT$^-$ 2S MEL cells with human fibroblasts containing an X-11 translocation, and then growing the hybrid cells in HAT selective medium. The X portion of the chimeric chromosome contains the HGPRT gene, permitting survival in HAT medium, and the 11 portion contains the beta-globin gene as demonstrated by Southern blot analysis. The DNA sensitivity and methylation patterns of the human globin genes in these hybrid cells is under investigation.

2) M11X-2 and -3 produce human beta-globin mRNA but no human beta-globin polypeptide. In conjunction with Dr. Joel Shapiro (see Project # Z01 HL 02217-01 MH), the putatively abnormal human beta-globin mRNA is being cloned and sequenced.

3) Transcription initiation factor activity has been detected in crude extracts of 2S MEL cells using the assay published by Roeder and his colleagues for identifying specific initiation on Ad-2 viral templates. Large scale purification procedures have been set up in order to identify, purify and characterize individual regulatory factors from the 2S MEL cells. One fraction, called Fraction A, is being purified to homogeneity.

Significance to Biomedical Research and Institute Program:

The molecular control of eukaryotic gene expression remains one of the major questions in biology today. Once it is better-understood how a gene in a eukaryotic cell is controlled, this knowledge can be applied to a wide range of human diseases including genetic diseases, viral diseases, etc.

Proposed Course of Project:

Fractionation of cell components from 2S MEL (and other) cells to identify, purify, and characterize individual regulatory factors involved in gene expression. 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 gene expression. The structure and function of the abnormal human beta-globin mRNA in the somatic cell hybrids will continue to be studied.

Publications:

1) Vembu, D., Young, N.S., Willing, M., Church, E., Sanders-Haigh, L., and Anderson, W.F.: Regulation of human globin gene expression in mouse erythroleukemia x human fibroblast hybrid cells. J. Cell Biol., in press, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02213-04 MH

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Regulation of Protein Biosynthesis in Cell-Free Systems

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

PI:	B. Safer	Medical Officer	MH NHLBI
OTHER:	R. Jagus	Visiting Associate	MH NHLBI
	D. Crouch	Biologist	MH NHLBI
	A. Konieczny	Visiting Fellow	MH NHLBI
	E. Church	Microbiologist	MH NHLBI

COOPERATING UNITS (if any) P. Cohen, University of Dundee, Dundee, Scotland; I. G. Wool, University of Chicago, Chicago, Illinois; P. Torrence, Laboratory of Chemistry, NIAMDD

LAB/BRANCH
Laboratory of Molecular Hematology

SECTION
Section of Protein Biosynthesis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 3.1	PROFESSIONAL: 1.4	OTHER: 1.7

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)

Regulation of protein synthesis at the level of mRNA translation is important for: (1) viral shut-off of host protein synthesis, (2) rapid adjustments to changing metabolic conditions, and (3) coordination of heme-globin-biosynthesis during red blood cell maturation. We have therefore studied the role of eIF-2 phosphorylation and mixed disulfide formation during protein synthesis initiation, and its regulation by the adenylate energy change, the pyridine nucleotide redox state and hemin, in reticulocyte lysate and intact cells. Translational regulation is shown to involve changes in the phosphorylation state and the oxidation/reduction state of the initiation factor eIF-2. In addition, a new polypeptide complex was isolated which participates in the catalytic utilization of eIF-2. The phosphorylation state of eIF-2 has been measured by chemical methods and the results correlated with the extent and kinetics of onset of translational inhibition. It now appears that phosphorylation of eIF-2 does not directly inhibit its activity but may interfere with the interaction of eIF-2 and the new polypeptide complex. Phosphorylation may promote additional modifications of eIF-2, however, which result in conversion from catalytic to stoichiometric utilization of eIF-2.

PHS-6040
(Rev. 2-81)

Objectives: The major goals are to (1) identify and characterize the covalent regulatory mechanisms which affect the rate and specificity of protein synthesis, (2) determine the sites and mechanisms of translational control of protein synthesis, (3) identify, isolate, and characterize the translational and regulatory components involved in such regulation, and (4) examine the participation of these mechanisms in the regulation of globin gene expression in normal and diseased states. We hope to develop methods which allow a quantitative determination of the number of regulatory sites and the extent of eIF-2 covalent modification under a wide variety of physiologic conditions by which the initiation of protein synthesis is regulated at the point of eIF-2-mediated methionyl-$tRNA_f$ binding.

Methods: A new purification procedure has been developed to allow the rapid isolation of the Met-$tRNA_f$ binding factor eIF-2 as part of a new macromolecular complex believed to participate in its catalytic recycling. A second approach being developed involves the use of monoclonal antibodies against eIF-2 and affinity chromatography. The oxidation/reduction state of cysteine residues will be quantitated through the use of highly specific fluorescent sulfhydryl group reagents. The phosphorylation state of eIF-2 in unfractionated lysate under different metabolic conditions will be determined by two-dimensional polyacrylamide gel electrophoresis or the isolation of eIF-2 using suitable precautions to eliminate eIF-2 kinase and phosphatase activity during the purification procedure. The phosphate content of the eIF-2α and β subunits resolved by high performance liquid chromatography (HPLC) is determined as alkalai-labile phosphate by the procedure of Ames. Specific pre-initiation complexes formed during the initiation of protein synthesis in normal and inhibited reticulocyte lysate are isolated using sucrose density gradient centrifugation. RNA components are quantitatively identified by nucleotide hybridization techniques. Radiolabeled aminoacyl- and peptidyl-tRNAs are determined as cetyltrimethylammonium bromide precipitate. Protein components are identified by direct or autoradiographic visualization of sucrose gradient fractions following resolution by polyacrylamide gel electrophoresis. Translational components are purified from these specific preinitiation complexes using ion exchange column chromatography, glycerol and sucrose density gradient centrifugation, gel filtration, and affinity column chromatography. Unfractionated messenger-RNA-dependent and gel-filtered reticulocyte lysate preparations, as well as purified translational components, are used in these studies.

Major Findings:

1) A second phosphorylation site on the α-subunit of eIF-2 has been identified following the isolation of eIF-2 under conditions which freeze the phosphorylation state of this factor during its purification. eIF-2α kinase, regulated by hemin, does not phosphorylate this new phosphorylation site. Following purification by CM cellulose chromatography and HPLC, the phosphate content of eIF-2α is 1.1 pmol phosphate/pmol subunit and that of eIF-2β is two pmol phosphate/pmol subunit. When lysate is incubated in the absence of hemin, the phosphate content of eIF-2β remains unchanged but the phosphate content of eIF-2α increases from 1.1 to 1.3 pmol phosphate/pmol subunit. The increase in eIF-2α phosphorylation occurs at the site phosphorylated by the hemin-regulated kinase. Under all conditions, no phosphate can be detected in eIF-2γ. Using eIF-2 preparations which have previously been totally dephosphorylated by eIF-2 phosphatase, we find that eIF-2β contains two phosphorylation sites which can be phosphorylated by a cAMP-

independent eIF-2β kinase. eIF-2α contains a single phosphorylation site which is specifically phosphorylated by the cAMP-independent eIF-2α kinase and a second site whose kinase remains unidentified.

2) The β-subunit of eIF-2 can be ADP-ribosylated by cholera toxin. This indicates that this subunit may have a GTP binding site which may be involved in the overall regulation of Met-tRNA$_f$ binding activity.

3) A new polypeptide complex has been isolated which may promote the catalytic utilization of eIF-2 during protein synthesis initiation. This recycling factor (RcF) has a native molecular weight of 400,000 daltons when associated with eIF-2. The addition of RcF, either alone or as a RcF·eIF-2 complex, to hemin-deficient lysate effectively restores protein synthesis initiation to control levels.

4) The mechanism of protein synthesis inhibition which results from the removal of low molecular weight components by gel filtration of lysate has been demonstrated to result from the conversion of eIF-2 utilization from a highly catalytic process to a stoichiometric one. Although biphasic kinetics of inhibition are observed, this inhibition of eIF-2 reutilization does not involve alteration of the phosphorylation state of the eIF-2α subunit. Rather, the oxidation of unpaired cysteine residues on the β and γ subunits of eIF-2 to form mixed eIF-2-glutathione disulfides appears to reduce the capacity of eIF-2 to be reutilized in subsequent rounds of initiation. The specific defect may involve a failure of eIF-2·GDP to dissociate upon release of eIF-2 from the 48S preinitiation complex.

5) The mechanism by which sodium selenite inhibits the initiation of protein synthesis has been determined. Sodium selenite is a unique sulfhydryl group reagent in that, in the presence of excess sulfhydryl group reagents, it spontaneously decomposes to elemental selenium, which is essentially inert. The addition of sodium selenite to unfractionated reticulocyte lysate can directly inactivate eIF-2 since this protein synthesis initiation factor contains sensitive sulfhydryl groups. In addition, the eIF-2α kinase is also activated by sodium selenite. Although the activity of eIF-2 inactivated by selenite can be restored by the addition of NADP-linked substrates which regenerate excess reduced glutathione, the activity of eIF-2α kinase remains elevated. This leads to an increased phosphorylation state of the eIF-2α subunit. Titration of selenite into reticulocyte lysate can produce a graded extent of eIF-2α phosphorylation. However, despite a six-fold difference in the extent of eIF-2α phosphorylation that can be achieved, the extent of translational inhibition is identical. The correlation that does exist between the extent of eIF-2α phosphorylation is that the time of onset for translational inhibition to occur is decreased the greater the extent of eIF-2α phosphorylation. This may indicate the presence of a multi-step mechanism of inhibition in which the initial phosphorylation of eIF-2α predisposes eIF-2 to undergo a second modification which then is responsible for the inhibition of eIF-2 and protein synthesis initiation.

6) The function of each of the three eIF-2α, β and γ subunits is being investigated using native preparations of eIF-2 which have been isolated containing only two of the three subunits normally found. We have found that the γ subunit of eIF-2 is responsible for binding Met-tRNA$_f$. The α-subunit of eIF-2, which undergoes phosphorylation by a specific, hemin-regulated protein kinase, appears to

regulate the activity of eIF-2 or its ability to recycle. The β-subunit of eIF-2 may be directly involved in the reactivation of eIF-2 and dissociation of GDP following its release from the 48S preinitiation complex.

Significance to Biomedical Research and Institute Program:

Although regulation at the level of transcription is currently thought to be the primary mechanism for regulating the flow of genetic information, modulation of protein synthesis has recently assumed increased importance. Translational regulation has been identified as a major feature of host-virus interaction. Regulation of protein synthesis by hemin was once thought to be a highly specialized means of coordinating globin chain and hemin synthesis in reticulocytes; it now appears, however, to be a widespread mechanism for post-transcriptional modulation of gene expression involving a cascade of highly specific protein kinases and other covalent modifiers. Final control of gene expression during cell differentiation may also be regulated by interaction of translational components with messenger RNA. It is essential, therefore, to understand the basic mechanism involved in these processes to be able to control gene expression in the cell.

Proposed Course of the Project:

Peptides containing the phosphorylation sites of eIF-2α and β will be isolated by HPLC and sequenced. The amino acid sequence will then be used to construct cDNA probes for the isolation of eIF-2 mRNA and to obtain its sequence by DNA-cloning techniques. This will allow more detailed studies of the molecular basis of the regulation of eIF-2 function by covalent as well as non-covalent mechanisms. Methodology for the production of monoclonal antibodies against protein initiation factors is also being developed. This will facilitate the study of initiation factor function in complex unfractionated biological systems.

Procedures for the identification and quantitation of covalent modifications of eIF-2 will also be improved and expanded to the study of other regulatory components. The overall goal of these approaches will be to understand the mechanisms of translational regulation in normal and disease states. In addition, these approaches will be extended to examine transcriptional regulation in *in vitro* systems currently being developed in the LMH.

Publications:

1. Safer, B., Jagus, B., Crouch, D., and Kemper, W.: Regulation of eIF-2 activity in reticulocyte lysate. In Gordon, J. and Thomas G. (Eds.): *Protein Phosphorylation and Bioregulation*. Basel, A. Karger, AG, 1980, pp. 142-153.

2. Crouch, D., and Safer, B.: Purification and properties of eIF-2 phosphatase. *J. Biol. Chem.* 255: 7918-7924, 1980.

3. Safer, B., Jagus, R., and Crouch, D.: Indirect inactivation of eIF-2 in reticulocyte lysate by selenite. *J. Biol. Chem.* 255: 6913-6917, 1980.

4. Stewart, A. A., Crouch, D., Cohen, P., and Safer, B.: Classification of an eIF-2 phosphatase as a type-2 protein phosphatase. *FEBS Lett.* 119: 16-19, 1980.

5. Safer, B., and Jagus, R.: New developments in the regulation of eIF-2. Biochimie, in press, 1981.

6. Safer, B., Jagus, R., and Crouch, D.: Both the redox and phosphorylation states of eIF-2 regulate protein synthesis initiation. Cold Spring Harbor Symposium on Quantitative Biology, in press, 1981.

7. Chan, Y.-L., Ulbrich, N., Ackerman, E. J., Todokoro, K., Slobin, L. I., Safer, B., Sigler, P. B., and Wool, I. G.: The binding of transfer ribonucleic acids to 5S and 5.8S eukaryotic ribosomal ribonucleic acid-protein complexes. J. Biol. Chem., in press, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02214-04 MH

PERIOD COVERED October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Molecular Cloning of Eukaryotic Globin Gene Sequences

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

PI:	W. French Anderson	Chief	MH NHLBI
OTHER:	P. Berg	Expert	MH NHLBI
	M. Huberman	Research Associate	MH NHLBI
	E. Schmader	Chemist	MH NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH Laboratory of Molecular Hematology

SECTION Section of Molecular Cloning

INSTITUTE AND LOCATION NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 3.1	PROFESSIONAL: 2.1	OTHER: 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 purpose of this project is to apply recombinant DNA and cloning technology to the isolation and analysis of genomic DNA sequences involved in the regulation of globin gene expression in humans and animals. Fragments of DNA relating to globin and thymidine kinase gene expression in humans and/or animals are isolated from genomic clones (constructed in this and other laboratories) and subcloned into plasmids for use in studies on eukaryotic gene expression. A prokaryote-eukaryote-prokaryote DNA transfer and recovery system has been developed which is proving useful in studying the regulation of gene expression in eukaryotes.

PHS-6040
(Rev. 2-81)

Objectives: The objective of this project is to establish and apply plasmid and bacteriophage recombinant DNA cloning systems for the isolation and analysis of human and animal genomic DNA sequences involving the regulation of globin gene expression.

Methods:

1. Molecular cloning is by use of the *in vitro* packaging system of bacteriophage lambda. In the cloning procedure, the *charon* 4A arms are ligated with the genomic DNA such that long linear concatamers of DNA result. This *in vitro* recombinant DNA can then be efficiently packaged into bacteriophage particles, resulting in a cloned library of greater than one million segments of genomic DNA. The library is then screened with radioactively labeled probes by well-established plaque hybridization methods.

2. Subcloning smaller segments of these clones is done into bacterial plasmid vectors (such as pBR313, pBR322, pBR325). The plasmid vector is digested with a suitable restriction endonuclease, mixed and ligated with the fragment to be subcloned and transformed into bacterial cells. Those colonies containing the required plasmid clone can be determined first by antibiotic resistant tests and secondly, by examination by restriction endonuclease and Southern blot analysis of their plasmid DNA on agarose gels.

Major Findings:

1) A prokaryote-eukaryote-prokaryote DNA transfer and recovery system has been used in which a human beta-globin gene was inserted into a bacterial plasmid, the plasmid containing the globin gene was microinjected into a mouse L cell where it multiplied and underwent recombinant events, and the same plasmid was then recovered (in the original as well as in mutant forms) by transformation of susceptible bacteria with DNA from the microinjected mouse cells. DNA was isolated from two microinjected clones and was used in bacterial transformation experiments. A number of unique plasmids were isolated and characterized. The structure of these plasmids helped lead to finding number two, below.

2) The structure of the microinjected genes in the genetically corrected cells has been determined to be long head-to-tail tandem repeats (concatamers). The size the concatamers appear to be is 100 kb or more. Efforts are now being made to isolate and characterize these concatamers. The microinjected L cell DNA has also been packaged in lambda and is now being studied to determine if the concatamers are totally or in part integrated into the mouse genome.

Significance to Biomedical Research and Institute Program:

The technique of recombinant DNA technology is a new and extraordinarily powerful technique for manipulating genetic material. Specific genes can be isolated, purified and characterized. The structure that these cloned genes have when they are replicated into genetically defective cells is important in understanding gene expression. Increased knowledge of the structural organization and sequence of normal and mutated human genes should provide further understanding into the mechanism of genetic diseases.

Proposed Course of the Project:

The prokaryote-eukaryote-prokaryote DNA transfer and recovery system will continue to be used to study the feasibility of transferring functional genes into mammalian cells.

Publications:

1. Anderson, W.F., Kretschmer, P.J., Sanders-Haigh, L., Killos, L., and Diacumakos, E.G.: Gene transfer and *in vitro* expression. In Stamatoyannopoulos, G. and Nienhuis, A.W. (Eds.): *Organization and Expression of Globin Genes* (Second Conference on Hemoglobin Switching). New York, Alan R. Liss, Inc., 1981, pp. 301-312.

2. Kretschmer, P.J., Bowman, A.H., Huberman, M.H., Sanders-Haigh, L., Killos, L., and Anderson, W.F.: Recovery of recombinant bacterial plasmids from *E. coli* transformed with DNA from microinjected mouse cells. *Nucleic Acids Res.*, in press, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02215-03 MH

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Etiology of Three Mouse Alpha-Thalassemias

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

PI:	J. Martinell	Staff Fellow	LMH NHLBI
OTHER:	W. French Anderson	Chief	LMH NHLBI

COOPERATING UNITS (if any) J. B. Whitney, Department of Cell & Molecular Biology, Medical College of Georgia, Augusta, GA 30912; R. A. Popp, Oak Ridge National Laboratory, Oak Ridge, TN 37830

LAB/BRANCH
Laboratory of Molecular Hematology

SECTION
Section of Molecular Genetics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Three induced mutations of the mouse have resulted in duplications of β-globin gene loci. We have studied the globin RNA levels in these mutant animals while the globin DNA structure and globin polypeptide synthesis levels of these mice are under investigation by Dr. R. A. Popp. Two of these mutations have been shown to have normal β-/α-globin polypeptide levels as well as β-/α-globin RNA levels in peripheral reticulocytes. These findings are similar to our earlier observations involving α-thalassemic mice. In the α-thalassemics, abnormal β-/α-globin gene ratios were not reflected precisely by the mRNA ratios; rather, the RNA ratios were nearer the normal level than were the β-/α-globin gene ratios. The third type of mouse has been found to have variable β-/α-globin polypeptide ratios (0.6 to 1.0) but constant β-/α-globin RNA ratios (0.7). This discrepancy is under further investigation.

PHS-6040
(Rev. 2-81)

Objectives: The objective of this work is to characterize at the molecular level mice which contain duplications of β-globin gene loci in order to understand better how globin gene expression is regulated in mammals. This work is being done in collaboration with Dr. R. A. Popp at Oak Ridge. Our specific objective is to measure the levels of α- and β-globin RNAs in the reticulocytes of these mutant mice.

Methods:

Preparations of total reticulocyte RNA from mutant mice were hybridized in solution to single-stranded [^{32}P]α- and [^{3}H]β-globin cloned cDNA probes. The hybridization kinetics of RNAs from mutant and normal (i.e., C57BL/6J) mice were then analyzed and compared.

Major Findings:

(1) Total reticulocyte RNAs from mice with duplications at β-globin loci were analyzed by liquid nucleic acid hybridization to cloned α- and β-globin probes. Mice with β-globin duplications have normal (i.e., 1:1) β-/α-globin RNA ratios compared to C57BL/6J mice. This finding is similar to our earlier results which indicated a nearly normal β-/α-globin RNA ratio in mice with deletions of α-globin loci (the α-thalassemic mice). We have inferred the action of one or more mechanisms that drive the β-/α-globin RNA ratios toward unity despite abnormal β-/α-globin gene ratios.

(2) Globin polypeptide synthesis ratios in intact reticulocytes of the mutant mice mentioned above are also normal. This work is being done by Dr. R. A. Popp at Oak Ridge.

(3) Total reticulocyte RNA hybridization analyses on an individual mouse (♀3695) which also contains duplicated β-globin loci have shown that the β-/α-RNA ratio is abnormal. A β-/α-globin ratio of 0.7 has been found consistently, even though variable β-/α-globin polypeptide synthesis ratios have been obtained. We do not yet understand the significance of this discrepancy; Dr. R. A. Popp is investigating this phenomenon further.

Significance to Biomedical Research and Institute Program: These observations are extensions of our earlier work involving thalassemic mice. These mice, together with thalassemic mice, are the best models currently available for a human molecular disease and are valuable models for the study of mechanisms of gene expression in general.

Proposed Course of the Project:

The nucleic acid measurements have been completed and Dr. J. Martinell has taken a new position outside this field. Dr. R. A. Popp is investigating further the globin gene structure and the globin polypeptide synthesis of these mice.

Publications:

1. Martinell, J., Whitney, J.B., III, Popp, R.A., Russell, L.B., and Anderson, W.F.: Three mouse models of human thalassemia. Proc. Natl. Acad. Sci., in press, 1981.

2. Whitney, J.B., III, Martinell, J., Popp, R.A., Russell, L.B., and Anderson, W.F.: Deletions in the alpha-globin gene complex in alpha-thalassemic mice. _Proc. Natl. Acad. Sci._, in press, 1981.

3. Popp, R.A., Lalley, P.A., Whitney, J.B., III, and Anderson, W.F.: Mouse α-globin genes and α-like pseudogenes are not syntenic. _Proc. Natl. Acad. Sci._, in press, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02216-02 MH

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Correction of Genetic Defects by Gene Transfer

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

PI:	W. F. Anderson	Chief	MH NHLBI
OTHER:	P. Berg	Expert	MH NHLBI
	S. Bernstein	Biologist	MH NHLBI
	J. DiPietro	Biologist	MH NHLBI
	L. Killos	Biol. Lab. Tech.	MH NHLBI
	K. Humphries	Visiting Associate	CH NHLBI
	A. Nienhuis	Chief	CH NHLBI

COOPERATING UNITS (if any)
E. G. Diacumakos, Ph.D., Rockefeller University, New York, New York
P. Hoppe, Ph.D. and J. Barker, Ph.D., The Jackson Labs, Bar Harbor, Maine

LAB/BRANCH
Laboratory of Molecular Hematology

SECTION
Section of Molecular Genetics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 2.7	PROFESSIONAL: 0.9	OTHER: 1.8

CHECK APPROPRIATE BOX(ES)
☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
Methods have been developed for transferring functional genes into mammalian cells. A primary technique is physical microinjection of specific cloned genes into the nucleus of individual tissue culture cells and into mouse zygotes. A genetic defect, lack of thymidine kinase (TK) activity, was corrected in mouse TK^- L cells by microinjecting a purified herpes simplex TK gene. A coinjected human beta-globin gene will replicate and express at a low level in these cells.

PHS-6040
(Rev. 2-81)

Objectives: The objective of this project is to develop methods for transferring functional genes into mammalian tissue culture cells and into intact animals. Ultimately, the techniques would be used for attempting to correct genetic diseases in human patients.

Methods:

1) Tissue culture cells are grown under standard tissue culture conditions.

2) Plasmids containing specific cloned genes are made by standard recombinant DNA techniques.

3) Physical microinjection into specific regions of intact mammalian cells is carried out by using ultra-thin micropipettes under 2,000-fold phase optics according to the procedure of Diacumakos (Diacumakos, E.G., Methods in Cell Biology 7: 287-311, 1973). Single cell clones are grown to 10^8-10^9 cells for isolation of DNA and RNA for analysis by restriction endonuclease and Southern blot analysis, by mRNA-cDNA liquid hybridization, or by Northern blot analysis.

Major Findings:

1) Purified DNA fragments containing the herpes simplex TK gene are effective in curing the genetic defect in mouse TK^- L cells. Thus, the plasmid sequences (contained in the recombinant plasmid pX1, previously used for correction of TK^- L cells) are not necessary for the genetic correction.

2) Preliminary indications imply that microinjection of fertilized mouse eggs with cloned genes might result in newborn mice which express the injected gene.

Significance to Biomedical Research and Institute Program:

The long-term aim of much of the work in molecular genetics is to develop techniques for treating or curing human genetic defects. This project utilizes recombinant DNA technology and cell biology techniques to try to accomplish this goal.

Proposed Course of the Project:

Analysis of genes microinjected into mouse embryos and into erythroid cells (viz. mouse erythroleukemia cells) will continue in order to understand regulation of the globin gene locus.

Publications:

1. Anderson, W.F., Killos, L., Sanders-Haigh, L., Kretschmer, P.J., and Diacumakos, E.G.: Replication and expression of thymidine kinase and human globin genes microinjected into mouse fibroblasts. Proc. Natl. Acad. Sci. USA 77: 5399-5403, 1980.

2. Anderson, W.F., and Fletcher, J.C.: Gene therapy in human beings. When is it ethical to begin? New Eng. J. Med. 303: 1293-1297, 1980.

3. Diacumakos, E.G., Killos, L., Lee, L., and Anderson, W.F.: Induction of mouse erythroleukemia cells by microinjection of inducing compound. Exptl. Cell Res. 131: 73-77, 1981.

4. Anderson, W.F., and Diacumakos, E.G.: Genetic engineering in mammalian cells. Sci. Amer. 245: 106-121, 1981.

5. Anderson, W.F., Killos, L., Sanders-Haigh, L., Kretschmer, P., Diacumakos, E., Nienhuis, A., Willing, M., and Vembu, D.: Regulation of human globin gene expression after gene transfer. Univ. of Chicago Symposia on Sickle Cell Anemia, Vol. 1 (Molecular Basis of Mutant Hemoglobin Dysfunction), Sigler, P.B. (ed.), North-Holland Publishing Co., New York, 1981, in press.

6. Anderson, W.F.: Expression of microinjected eukaryotic genes. Second International Symposium on RNA Development and Reproduction. Peking, China, 1981, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02217-01 MH

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Chemical Synthesis and Molecular Cloning of Eukaryotic Gene Sequences

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

PI:	Joel Shapiro	Staff Fellow	MH NHLBI
OTHER:	W. F. Anderson	Chief	MH NHLBI
	B. Safer	Medical Officer	MH NHLBI
	Y. Chiang	Chemist	MH NHLBI

COOPERATING UNITS (if any)
Dr. H. Bryan Brewer, Molecular Disease Branch, NHLBI; Dr. F. Thomas, Molecular Disease Branch, NHLBI; Dr. W. Lovenberg, Hypertension-Endocrine Branch, NHLBI

LAB/BRANCH
Laboratory of Molecular Hematology

SECTION
Section of Molecular Cloning

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 1.4	PROFESSIONAL: 1.2	OTHER: 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 intent of this project is to apply chemical oligonucleotide synthesis, recombinant DNA technology, and cell biology techniques to the isolation, analysis, and characterization of normal genes corresponding to genetic diseases. The enzymes phenylalanine hydroxylase and hypoxanthine guanine phosphoribosyl transferase have been isolated in order to obtain amino acid sequencing information. Chemical synthesis of oligonucleotides has been established. An octadecanucleotide complementary to a portion of the human β-globin mRNA has been synthesized for use in analysis of β-globin regulation. Eight pentadecanucleotides which potentially complement a portion of the mRNA encoding rat phenylalanine hydroxylase have also been synthesized. Total cell mRNA has been prepared from rat liver, human reticulocytes, mouse, and hybrid (human/mouse) cells.

PHS-6040
(Rev. 2-81)

Objectives: The objectives are to: (1) purify and sequence protein products which, when deficient, are manifest as genetic diseases; (2) establish chemical synthesis of oligo- and polynucleotides (DNA); (3) apply the use of synthetic oligonucleotides to identify, isolate (or synthesize), clone and characterize genes encoding for normal function which, when aberrant, lead to a diseased state; and (4) correct the diseased state in tissue culture via introduction of the normal gene. Ultimately, the major goal is to treat or cure genetic diseases in humans.

Methods:

1) Protein purification is achieved by use of affinity chromatography using phenyl Sepharose (phenylalanine hydroxylase - PH) or GMP Sepharose (hypoxanthine guanine phosphoribosyl transferase - HGPRT), in conjunction with ion exchange chromatography, molecular sieve chromatography, membrane ultracentrifugation, and ammonium sulfate precipitation. Verification of protein identity is done using *in vitro* enzyme assays. PH is assayed using a spectrophotometric determination of phenylalanine conversion to tyrosine. HGPRT activity is determined using a novel, rapid and quantitative high performance liquid chromatography (HPLC) assay for conversion of guanine to GMP. Further confirmation of protein identities is obtained through SDS polyacrylamide molecular weight comparison with literature values. Purification of CNBr peptide fragments is being performed using reversed-phase HPLC.

2) Oligonucleotide synthesis is performed using the modified triester approach in solution phase using block condensations with intermediary silica gel purifications. Following removal of blocking groups used in the synthesis, the oligonucleotides are purified using ion-exchange HPLC and Sephadex gel filtration HPLC. These molecules are tested for biological activity *in vitro* as substrates for polynucleotide kinase.

3) mRNA is isolated from rat liver and human reticulocytes using phenol extraction followed by 2X chromatography on oligo(dT)-cellulose. mRNA is also isolated from M11X (human/mouse hybrid) cells, and 2S MEL (mouse) cells using CsCl centrifugation followed by 2X chromatography on oligo(dT)-cellulose. *In vitro* translation of the isolated total mRNA preparations using the rabbit reticulocyte lysate system is used to verify the presence of translatable mRNA. Acid urea polyacrylamide slab gel analysis of the *in vitro* translation products of human reticulocyte, M11X, and 2S mRNA preparations is used to determine if mRNA encoding for β-globin (mouse or human) is present.

Major Findings:

1) Phenylalanine hydroxylase (PH) has been purified from rat, monkey, and human tissue. Protein identification was based upon SDS polyacrylamide gels and an *in vitro* spectrophotometric assay for phenylalanine conversion to tyrosine. Hypoxanthine guanine phosphoribosyl transferase (HGPRT) was isolated from human blood. HGPRT activity was measured using a novel HPLC assay. Lack or reduction in the activity of both enzymes results in genetic diseases; phenylketonuria is the result of lack of PH; Lesch-Nyhan syndrome and some forms of gout are caused by lack or reduction in HGPRT activity. Both intact proteins are N-terminally blocked and thus refractile to direct sequence analysis. In the case of HGPRT,

CNBr cleavage at methionine residues is being employed to obtain fragments suitable for sequence analysis.

2) Chemical synthesis of oligonucleotides has been established using the modified triester approach. Dinucleotide and trinucleotide blocks were synthesized and subsequently joined in solution to form oligonucleotides. Based upon a published partial amino acid sequence for rat PH and the multiple nucleotide triplets encoding for various amino acids, eight pentadecanucleotides were synthesized which potentially correspond to a portion of the mRNA encoding for rat PH. An octadecanucleotide complementary to a portion of the human β-globin mRNA has also been synthesized.

3) For the purpose of isolation of unique mRNA corresponding to a specific gene product, sequence analysis, cDNA production, and isolation and cloning of the genomic DNA, mRNA was prepared from rat liver, human reticulocytes, 2S MEL (mouse) cells in tissue culture, and M11X (human/mouse hybrid) cells in tissue culture. The preparations were tested for in vitro translation using the rabbit reticulocyte lysate system. Confirmation of globin mRNA in the human reticulocyte, 2S MEL and M11X cells was obtained using acid-urea polyacrylamide slab gels.

Significance to Biomedical Research and Institute Program: The use of synthetic oligonucleotides will allow for the isolation and cloning of any gene whose sequence, mRNA sequence, or protein sequence is determined. Coupled with a further understanding of gene regulation (including the β-globin work described below), recombinant DNA technology, and cell biology techniques, the treatment or curing of human genetic diseases may be obtained.

Proposed Course:

1) Utilizing peptide sequence information obtained from CNBr-cleaved HGPRT fragments, synthetic oligonucleotides will be constructed in order to sequence the mRNA, clone the cDNA and clone the genomic DNA. The cDNA and genomic DNA clones will be used to attempt to correct for HGPRT deficiency in tissue culture cells.

2) The β-globin specific primer will be used to purify β-globin mRNA from the total cell mRNA populations isolated from human and M11X cells and to synthesize cDNA. Sequence analysis may provide insight into β-globin regulation. If sequence differences between human and M11X globin mRNA are observed, the genomic DNA will be isolated and compared. If no difference in cDNA sequence is observed, the mechanism of post-transcriptional modification will be pursued.

3) Chemical oligonucleotide synthesis capabilities will be expanded to include solid-phase chemistry utilizing both the triester and phosphite approaches. Enzymatic oligonucleotide synthesis will also be explored.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02218-01 MH

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Regulation of eIF-2 Activity: Model Phosphoprotein and GTP-Modulated Regulatory Protein

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

PI:	Rosemary Jagus	Visiting Associate	MH NHLBI
OTHER:	Brian Safer	Medical Officer	MH NHLBI
	Andrzej Konieczny	Visiting Fellow	MH NHLBI
	Deborah Crouch	Biologist	MH NHLBI
	Eve Church	Microbiologist	MH NHLBI

COOPERATING UNITS (if any)
Dr. Dermot Cooper, Lab. Nutrition & Endocrinology, NIADDK; Dr. Robert L. Somers, Lab. Vision Research, NEI; Drs. Peter Nielson and Julian Gordon, Friedrich Miescher Institute, Basel, Switzerland

LAB/BRANCH
Laboratory of Molecular Hematology

SECTION
Section of Protein Biosynthesis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 1.8	PROFESSIONAL: 1.5	OTHER: .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)

Eukaryotic initiation factor 2, eIF-2, plays a regulatory role in the initiation of protein synthesis in animal cells. eIF-2 activity is modulated by phosphorylation, guanine nucleotides and the redox state of its sulfhydryl groups and has been used as a model protein in which to study these three important regulatory mechanisms of animal cell function. eIF-2 is an allosteric protein whose activity is modulated by sequential changes in its conformation to fulfill its function at different stages of its activity cycle. Phosphorylation of the α-subunit does not directly reduce its Met-$tRNA_f$ binding ability, but prevents one of the sequential conformational transitions such that the β-subunit of eIF-2 is unable to interact with other components of the eIF-2 activity cycle.

PHS-6040
(Rev. 2-81)

Objectives: The major objectives are to: (1) identify, isolate and characterize components involved in the eIF-2 activity cycle; (2) elucidate the effects of phosphorylation, guanine nucleotides and changes in the redox state of sulfhydryl groups on the activity cycle of eIF-2; and (3) compare eIF-2 with other GTP-modulated regulatory proteins.

Methods: The phosphorylation state of eIF-2 is determined by isolation of eIF-2 under conditions chosen to preserve the in situ phosphorylation state. The phosphate content of the α-subunit of eIF-2, resolved by reverse phase HPLC, is determined as alkali-labile phosphate by the procedure of Ames. ADP-ribosylation by cholera toxin and NAD is performed essentially as described by Kaslow et al. (1979) (Mol. Pharmacol. 15: 472) and the proteins separated by SDS/PAGE. The fate of eIF-2 and other components of initiation during changes in protein synthetic rate is monitored by sucrose density gradient centrifugation. RNA components are identified and quantitated by hybridization techniques and cetyltrimethylammonium bromide precipitation. Protein components are identified by direct or autoradiographic visualization of sucrose gradient fractions following resolution by SDS/PAGE. An eIF-2-dependent cell-free protein synthesizing system has been developed in which to estimate the activity of modified eIF-2. Monoclonal antibodies to eIF-2 are being developed. Detection of eIF-2 antibody-producing hybridomas includes the electrophoretic blotting procedure of Towbin et al. (1979) (PNAS 76: 4350), ELISA and radioimmunoassay procedures.

Major Findings:

1. When reticulocyte lysate is incubated in the absence of hemin, there is an increase in phosphorylation of the α-subunit of eIF-2 at the site phosphorylated by the hemin-regulated kinase. However, the steady-state level of phosphorylation at this site is only 25-30% of the available eIF-2 pool.

2. The isolation of eIF-2 under the same conditions described above has resulted in the identification of a second phosphorylation site on the α-subunit of eIF-2. This site is not phosphorylated by the hemin-regulated kinase and seems fully occupied in lysate incubated in the presence or absence of hemin.

3. The β-subunit of eIF-2 can be ADP-ribosylated by cholera toxin which converts it to a form which cannot be utilized catalytically and which implicates the β-subunit in playing a role in the recycling of eIF-2 and dissociation of GTP following the release of eIF-2 from the 48S preinitiation complex. In addition, other GTP-modulated regulatory proteins were examined including adenylate cyclase from various sources and retinal GTPase which is involved in the rhodopsin-linked activation of phosphodiesterase. The factors all contain components which are ADP-ribosylated by cholera toxin which interacts with guanine nucleotide binding sites. The function of adenylate cyclase is also modified by ADP-ribosylation. These results imply a degree of conservation of structure of these GTP-modulated regulatory proteins, hint at a common evolutionary origin and suggest the proteins may share immunoreactivity.

4. The selective removal of small molecular weight components of reticulocyte lysate by gel filtration has resulted in the generation of lysate with varying functional characteristics including hemin-independent and eIF-2-dependent lysate. These have provided ideal assay systems in which to study agents or modifications which affect eIF-2 activity. Use of these lysates established that eIF-2 activity

could be regulated at the level of reutilization of eIF-2 released from preinitiation complexes.

5. In the absence of hemin, eIF-2 is released prematurely from preinitiation complexes by a salvage mechanism involving a ribosome-bound Met-$tRNA_f$ deacylase. This premature release seems to occur because eIF-2 with a phsophorylated α-subunit (hemin-regulated kinase site) is unable to undergo a conformational transition enabling it to complete the final stage of initiation.

6. A hybridoma cell line has been established which produces monoclonal antibodies to the β-subunit of eIF-2.

Significance to Biomedical Research and Institute Program:

Translational regulation represents the short-term regulation of gene expression found in animal cells and the mechanisms available are exploited during development, differentiation, transformation and by many animal cell viruses, making it important to understand the basic mechanisms involved. GTP-modulated regulatory proteins are an increasingly common element in animal cell regulation. Such proteins play roles in the hormonal control of adenylate cyclase, in information amplification in the visual response to rhodopsin and the maintenance of the transformed state by retroviruses. eIF-2, in its regulatory role in initiation, provides a useful model system in which to study modulation by guanine nucleotides.

Proposed Course of Project:

1. It is proposed to determine by peptide mapping and immunological studies the degree of relationship between the ADP-ribosylated peptides from eIF-2, adenylate cyclases from various membrane preparations, and the rhodopsin-linked GTPase.

2. Elucidation of the mechanism by which phosphorylation of eIF-2 leads to the inhibition of protein synthesis has been hampered by uncertainties concerning eIF-2 subunit composition and function, the size of the eIF-2 pool, an imperfect understanding of eIF-2 distribution under different protein synthetic conditions, and a lack of appreciation of the proteins with which eIF-2 normally associates in vivo. Monoclonal antibodies, currently being developed, are expected to resolve these questions.

Publications:

1. Jagus, R., Anderson, W.F., and Safer, B.: Initiation of mammalian protein synthesis. Prog. Biophys. Mol. Biol. 25: 127-185, 1981.

2. Jagus, R., and Safer, B.: Activity of eukaryotic initiation factor 2 is modified by processes distinct from phosphorylation. I. Activities of eukaryotic initiation factor 2 and eukaryotic initiation factor 2α kinase in lysate gel filtered under different conditions. J. Biol. Chem. 256: 1317-1323, 1981.

3. Jagus, R., and Safer, B.: Activity of eukaryotic initiation factor 2 is modified by processes distinct from phosphorylation. II. Activity of eukaryotic initiation factor 2 in lysate is modified by oxidation-reduction state of its sulfhydryl groups. J. Biol. Chem. 256: 1324-1329, 1981.

4. Jagus, R., Crouch, D., Konieczny, A., and Safer, B.: The role of phosphorylation in the regulation of eukaryotic initiation factor 2 activity. Current

Topics in Cellular Regulation, in press, 1981.

5. Cooper, D.M.F., Jagus, R., Somers, R., and Rodbell, M.: Cholera toxin modifies diverse GTP-modulated regulatory proteins. Nature, in press, 1981.

Annual Report of the
Section on Laboratory Animal Medicine and Surgery
Office of the Director of Intramural Research
National Heart, Lung, and Blood Institute
October 1, 1980 to September 30, 1981

The Section functions primarily in a support role to all laboratories of IR providing care for many species of animals, technical assistance in preparation and maintenance of animal models for various experimental regimens, and the development of animal resources not otherwise available.

Maintenance of various small animal species has been accomplished in designated areas in close proximity to IR laboratories in Buildings 3, 10, and 36. Large animal species are maintained in Buildings 3, 28, the NIHAC, and at Luray, Virginia. Postoperative intensive care and treatment of surgery patients is completed in Buildings 3, 14-E, and 28.

The animal surgery laboratory located in Building 14-E supports the Clinical Hematology Branch, Laboratory of Experimental Atherosclerosis, Hypertension-Endocrine Branch, Laboratory of Technical Development, the Surgery Branch, and the Pulmonary Branch in preparation of experimental animal models, completing cardiovascular studies and in collecting various biological specimens. The laboratory operates an x-ray catheterization suite, clinical chemistry laboratory, sterile operating suites, and special study suites required to meet IR requirements.

The NHLBI Sheep Colony continues year-round breeding of laboratory sheep. Approximately 500 animals were delivered to laboratories meeting requirements of gestation stages from 22-140 days and various age and size lambs, young adults, and aged sheep. In addition, postoperative animal models have been returned and maintained at the colony and more than 80 tons of feed supplies have been delivered to NIH.

Laboratory studies have been underway to define hemodynamic parameters and tissue morphology resultant of Newfoundland dog left ventricular hypertrophy due to infracoronary left ventricle outflow tract obstruction. Disease processes will be characterized to determine usefulness of this unique animal model for future laboratory study.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03401 -05 LAMS

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Newfoundland Breeding Colony

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

PI:	J. E. Pierce	Chief	SLAMS	NHLBI
	M. Jones		SB	NHLBI
Other:	D. K. Buckhold		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
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	3.0	1.0

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The Newfoundland Breeding Colony has been developed as a source of laboratory dogs affected with left ventricular hypertrophy (LVH) due to infracoronary left ventricle outflow tract obstruction and other spontaneously occurring heart defects. More than 100 dogs have been reared, all having some form of hereditary subaortic stenosis (SAS), with one family demonstrating pulmonic valve stenosis and SAS.

Numbers of animals have been used for regional myocardial blood flow studies of the hypertrophied ventricle and evaluation of corrective surgery techniques using apico-aorta prosthetic devices.

PHS-6040
(Rev. 2-81)

Project Description: Z01-HL-03401-05-LAMS

The Newfoundland Breeding Colony was maintained by Flow Laboratories, Inc., at Dublin, Virginia supported by NIH 263-78-D-0253. Twenty-eight dogs remain and are located in Building 28, including 14 males and 14 females with varying degrees of SAS and PAS.

Availability of a naturally occurring animal model for study of LVH resultant of infracoronary LV outflow tract obstruction is important because technical difficulties have not been satisfactorily overcome in attempts to produce such a defect in normal animals.

Discrete subaortic stenosis has been studied in Newfoundland dogs at the School of Veterinary Medicine, University of Pennsylvania where initial breeding experiments suggested that it is inherited and either a polygenic or an autosomal dominant trait with modifiers.

Breeding will continue to produce offspring for studies of mechanisms of early development of the obstructive subaortic ring.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER
		Z01 HL 03402-05 LAMS

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

NHLBI Laboratory Sheep Colony

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

PI:	J. E. Pierce	Chief	SLAMS	NHLBI

COOPERATING UNITS (if any)

1. Laboratory of Biomedical Sciences, IRP, NICHHD
2. ACS, VRB, DRS

LAB/BRANCH
Office of Director of Intramural Research

SECTION
Section on Laboratory Animal Medicine and Surgery

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.8	0.2

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The laboratory Sheep Colony is an NIH animal resource providing varied age animals that meet specific year-round requirements of the Clinical Hematology Branch, Laboratory of Technical Development, Pulmonary Branch, and Surgery Branch, DIR, NHLBI; and the Laboratory of Biomedical Sciences, IRP, NICHHD. Maintenance regimens in use have resulted in successful year-round breeding and production of varied age sheep.

Practices that have contributed to reduction of undesired seasonal variables include: (1) continuous prophylactic immunization of all age animal groups; (2) accurate pregnancy diagnosis during first trimester using Doppler ultrasound; (3) monitoring of animal health using various diagnostic laboratory techniques; and (4) many husbandry techniques unique to this colony. Such practices have been cost prohibitive in commercial sheep flocks that result in inconsistent availability and existence of varied states of health in animals delivered for laboratory use.

PHS-6040
(Rev. 2-81)

The breeding colony continues as a source of sheep with A, AB, and B type hemoglobin for the CHB and meets other supportive laboratory program requirements of young lambs and pregnant ewes as required. From 700 to 950 varied age sheep exist in the colony year-round. Approximately 500 animals were delivered to NIH and other facilities for laboratory use during the report period.

The contractor has been responsible for developing and updating husbandry techniques as instructed by the project officer that allow optimal conditions for natural year-round breeding at the contract site. The project officer has been responsible for monitoring techniques and updating guidelines followed by the contractor to meet NIH laboratory requirements along with animal health regimens (sheep property of NIH) that allow minimal susceptibility of all age groups to common sheep diseases.

Immunization protocols direct contract personnel to administer specific toxoids and bacterins to lambs with biweekly boosters of each by the fifth week of age. This practice has essentially eliminated enterotoxemia and more than 90% of chronic pneumonia previously experienced. Repeated administration of indicated biologicals is carried out in all age groups at designated periods of development and production.

The necessity of an accurate method of pregnancy diagnosis during early gestation was determined during initial development of the colony. Natural seasonal and environmental conditions effect the conception rate varying from 0-100%. Lindahl's technique using Doppler ultrasound with rectal examination has been adequate. It allows accurate diagnosis of up to 100% of pregnant sheep from 21-35 days gestation. Negative animals must be re-examined at least twice to verify non-pregnancy. Examinations are performed weekly by contract personnel with more than 1,500 examinations completed per year to detect approximately 500 pregnant ewes conceived over a 52-week period.

Laboratory tests are continuously performed to monitor flock health. Contract personnel monitor internal parasite infestation by random fecal sampling from various animal groups with examination using direct and flotation methods. CBC and blood chemistry profiles are performed on animals delivered to the laboratory to monitor health and nutrition status. Microbiological and serological screening for detection of suspected disease entities is carried out when indicated.

In addition to producing sheep for laboratory use, more than 60 sheep prepared for study were returned to the contract site for maintenance for periods over 6 months and returned for study.

Also, more than 80 tons of feed supplies were delivered to NIH to allow continuation of colony animal diets in laboratory facilities.

This project will continue as IR and other NIH programs have sufficient requirements that justify the continued support of this laboratory sheep resource. Production goals and total numbers of animals maintained will be varied as required by changing demands of laboratories.

Contract Information:

Contract Number: 263-80-C-0007 - approximately $265,000 - 10/1/80 - 9/30/81

Contract Site: White House Farms, Inc.
Rt. 1, Box 403-E
Luray, Virginia 22835

PI: Max Foltz, Contractor
Rick Miller, Colony Manager

Total Manyears: 8.5

Professional: 2.0

Other: 6.5

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03403-04 LAMS

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Indirect Blood Pressure Measurements in Laboratory Animals Using Oscillometry.

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

PI:	J. E. Pierce	Chief	SLAMS	NHLBI
	E. Walker		BEI	DRS

COOPERATING UNITS (if any)

E. Walker, BEI, DRS

LAB/BRANCH
Office of Director of Intramural Research

SECTION
Section on Laboratory Animal Medicine and Surgery

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	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)

A technique for obtaining indirect blood pressure measurements in laboratory animals currently being evaluated, oscillometry, monitors the oscillations in cuff pressure with a pressure transducer as the cuff is deflated. During deflation from super systolic levels, the first increase in oscillation observed is designated as systolic blood pressure.

Mean arterial measurements obtained on anesthetized dogs with direct pressure measurements available were consistently within 1-3% of direct pressure.

Measurements obtained on awake dogs were not as consistent but corresponding values were obtained in both forelegs over a 15-minute examination period within 5% of each other. (Direct measurements not simultaneously available).

The technique will be further evaluated using a commercially available unit and a laboratory designated oscillometer which provides raw oscillation data to assist in determining optimal cuff sizes to be used and identify other variables that effect obtaining accurate blood pressure measurements in laboratory animals.

PHS-6040
(Rev. 2-81)

Project Description:

A technique for obtaining accurate indirect blood pressure measurements in laboratory animals, particularly dogs and miniature swine has been continuously requested by investigators of the Laboratory of Experimental Atherosclerosis, Hypertension-Endocrine Branch, and Surgery Branch, IR.

A technique using Doppler ultrasound with transducer secured to an inflatable cuff has proved unsatisfactory. Inconsistent blood pressure values were obtained in awake and anesthetized animals locating the cuff at various sites on the forelimb, rear leg, and tail as reported successful by other groups.

Because of the unreliability of traditional techniques, we have been investigating a technique called oscillometry. Oscillometry is a method of measuring blood pressure by analyzing the pulse pattern of the cuff pressure oscillations.

In practice, a cuff is placed around a limb and inflated to a pressure above systolic and then slowly deflated. While the cuff is being deflated the amplitude of oscillation in cuff pressure, produced by the arterial pulse beneath the cuff, is monitored. Systolic pressure is indicated by the first significant increase in oscillation amplitude. Diastolic pressure is indicated as the lowest cuff pressure at the point of maximum oscillation in cuff pressure. Currently, most investigators using this technique take the point of maximum oscillation to indicate mean arterial pressure.

The results of 19 dog experiments and many laboratory simulations indicate that oscillometry measures systolic and diastolic pressures but not the mean arterial pressure. The mean arterial pressure can be incorrectly indicated when there are unknown errors present in the technique.

Results of earlier studies and several additional will allow completion of this study.

ANNUAL REPORT OF THE
SECTION ON THEORETICAL BIOPHYSICS
OFFICE OF THE DIRECTOR OF INTRAMURAL RESEARCH
NATIONAL HEART, LUNG, & BLOOD INSTITUTE
October 1, 1980 through Setember 30, 1981

The primary interest of the Section on Theoretical Biophysics is the theory of transport processes in biological systems, with particular reference to problems in cardiovascular, renal, and membrane physiology. The section is concerned both with the formulation of theoretical models and with the development of mathematical and computational methods for their analysis. Currently much of the research in the section centers on the mechanism of urine formation in the mammalian kidney and on theoretical aspects of solute and water transport in epithelial structures.

During the past year, areas of work have included: (1) The thermodynamic and kinetic analysis of 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, (6) the computer simulation of solute and water transport in simple epithelia, and (7) the development of analytical models of epithelia.

The existence of multiple steady states for certain models of the renal medulla has been recognized by us for some time. Methods for locating and classifying these states have been devised (R. Mejia, A Path Following Method for Obtaining a Connected Component of the Solution Surface of a Convection-Diffusion Model in Fluid Flow, abstract, Annual Fall Meeting of the Maryland-District of Columbia-Virginia section of the Mathematical Association of America, November 1980). A method for obtaining such solutions for a multinephron kidney model and for studying the transition from one state to another as a function of renal parameters has been described (R. Mejia and J. L. Stephenson, Multiple Solutions of Convection-Diffusion Equations Describing the Renal Concentrating Mechanism, Numerical Methods for Engineering, GAMNI 2, 2nd International Congress, 1003-1013, 1980). The main conclusions of these investigations are (1) multiple stable steady states appear to be physiologically plausible; (2) such states are connected by hysteresis loops that might stabilize operation in substantially different regimes; (3) some paths, e.g., as a function of certain model parameters, are forbidden. These simulations have suggested that appropriate experiments might give evidence of multiple states.

An interactive continuation algorithm called SCOUT due to H-O. Peitgen, H. Jurgens and D. Saupe at Universtitat Bremen, has been implemented on the PDP-10 and PDP-11 computers at the NIH

Computer Center. It consists of a set of simplicial continuation utilities that accept a simple, FORTRAN, description of the set of equations whose roots are sought. It will homotope to a root from an initial guess; it will continue as a function of a parameter; allows for a variable mesh size; and uses a perturbation technique to resolve bifurcation points or seek disjoint solution branches. SCOUT has been used to map the solution surface for some analytic models and has served to verify results obtained for some small (up to 15 finite difference equations) discretized models using methodology described below.

SCOUT, although at the forefront of the state-of-the-art in numerical continuation, cannot begin to handle the nonlinear systems with hundreds of unknowns that arise in multinephron kidney models. Hence, the (partitioned) algorithm used to solve the time dependent kidney equations for many nephrons (R. Mejia and J. L. Stephenson, Numerical Solutions of Multinephron Kidney Equations, J. Comput. Phys. Vol. 32, Number 2, 235-246, 1979) has been extended. An adaptive multigrid method for solution of a multipoint boundary value problem has been developed. The $O(h^2)$ approximation used to solve the tubular equations in the partitioned algorithm has been replaced by an $O(h^4)$ scheme (R. P. Tewarson, On the Use of Simpson's Rule in Renal Models, Math. Biosci., to appear). These have been solved symbolically (R. Mejia and J. L. Stephenson, Symbolics and Numerics of a Multinephron Kidney Model, 1979 MACSYMA User's Conference, Washington, D. C., 596-603). The interstitial variables and the boundary equations are discretized using a difference scheme with space mesh size of h/2, which is conservative. The partitioned system is then solved using an adaptive, alternating method (R. Mejia and J. L. Stephenson, An Alternating Method of Solution of a Multipoint Boundary Value Problem, SIAM 1979 Fall Meeting, Denver, Colorado). Tests indicate that the increased accuracy in conjunction with a coarser mesh will permit solution of models with several nephron populations, something that is required, for example, in the study of multisolute systems.

Work has continued on approximate analytic solution of central core models of the renal medulla. Earlier analytic work has been extended by deriving general mass balance equations for an n-stage single solute central core model of the renal medulla. When these equations are combined with a previously derived approximate relation between axial volume flow, concentration, solute permeability, hydraulic permeability, and reflection coefficient; we are led to implicit equations for the concentration at the various medullary levels. For selected cases these can be solved to give a closed analytic solution, but in general they must be solved numerically. To do this and to explore the parameter space of these models, we have used the continuation algorithm called SCOUT described above. The results of these studies are now being written up for publication.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03201-21 STB

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Mathematical Theory of Renal Function

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

PI:	J. L. Stephenson	Chief, Section on Theoretical Biophysics	OD NHLBI
OTHERS:	R. Mejia	Mathematician	OD NHLBI
	B. Kellogg	Prof. IPST, University of Maryland	
	J. Garner	Prof. Louisiana Tech University, Ruston, La.	

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 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.7	1.2	.5

CHECK APPROPRIATE BOX(ES)
☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The purpose of this project is to develop the general theory of transport and flow processes taking place in the kidney. Aims of current work include (1) Thermodynamic and kinetic analysis of flow processes, (2) the qualitative analysis of equations describing kidney models, and (3) the development of analytical solutions of kidney models.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: The primary purpose of this project is to develop the general theory of the transport and flow taking place in the kidney. This includes the thermodynamic and kinetic analysis of flow processes; the development of analytical solutions of kidney models, and the qualitative analysis of equations describing kidney models.

During the past year, analytic work on n-stage models has continued.

(1) A simplified approach to mass balance in a single stage of a multistage model has been formulated:

Since transmural water and solute fluxes must cancel when summed over all medullary structures, it follows that both axial solute and volume flow along the medulla must be constant and equal to final urine volume flow and solute excretion. If we ignore axial diffusion, we have

$$\Sigma\ F_{iv}(x)\ C_{iM}(x) = F_{3v}(L)\ C_{3M}(L) \tag{1}$$

$$\Sigma\ F_{iv}(x) = F_{3v}(L) \tag{2}$$

If we introduce the subscripting convention DHL = 1, AHL = 2, CD = 3, AVR = 4, DVR = 5 and refer all solute concentrations to DVR, e.g.

$$C_{DHL} \equiv C_{DVR} + \xi_{DHL} \tag{3}$$

We can write

$$[F_{1v}(x) + F_{3v}(x) + F_{4v}(x) + F_{5v}(x)]C_{DVR}(x)$$
$$= F_{3v}(L)C_{3M}(L) - F_{2v}(x)C_{2M}(x) + T_W(x), \tag{4}$$

where $T_W(x) = -\sum_{i\neq 2} F_{iv}(x)\xi_i(x)$. Clearly the principal term in this sum is $F_{AVR}(x)\xi_{AVR}(x)$. Accordingly we will write

$$T_W(x) \cong - F_{AVR}(x)\ [C_{AVR}(x) - C_{DVR}(x)] \tag{5}$$

(It will be recalled that according to our sign convention, flow is positive toward the papilla.)

We also have

$$F_{1v}(x) + F_{3v}(x) + F_{4v}(x) + F_{5v}(x) = F_{3v}(L) - F_{2v}(x). \tag{6}$$

If we consider a single stage of a multistage model, volume flow in the AHL system is nearly constant, so for $x_1 \leqslant x \leqslant x_2$

$$F_{1v}(x) + F_{3v}(x) + F_{4v}(x) + F_{5v}(x) = F_{3v}(L) - F_{2v}(x_2). \qquad (7)$$

Equation (7) has the simple interpretation that in a region in which few or no DHL turns to become an AHL, the combined volume flow in DHL, CD, and vasa recta is nearly constant and equal to the sum of the final urine flow and the flow in the AHL system at the given level.

If we substitute (7) into (4) for the levels x_2 and x_1 defining the stage and substract, we obtain

$$\begin{aligned}
[F_{3v}(L) - F_{2v}(x_2)][C_{DVR}(x_2) - C_{DVR}(x_1)] \qquad (8) \\
= - F_{2v}(x_2)\, C_{2M}(x_2) + T_W(x_2) \\
+ F_{2v}(x_1)\, C_{2M}(x_1) - T_W(x_1) \\
= - F_{2v}(x_2)\, [C_{2M}(x_2) - C_{2M}(x_1)] \\
+ T_W(x_2) - T_W(x_1)\ .
\end{aligned}$$

[Note $F_{2v}(x_2) = F_{2v}(x_1)$].

This equation can be rewritten

$$C_{DVR}(x_2) - C_{DVR}(x_1) \qquad (9)$$

$$= \frac{- F_{2v}(x_2)[C_{2M}(x_2) - C_{2M}(x_1)] + T_W(x_2) - T_W(x_1)}{F_{3v}(L) - F_{2v}(x_2)}\ .$$

Equation (9) is nearly intuitively obvious: $C_{DVR}(x_2) - C_{DVR}(x_1)$ is the change in concentration in the DVR, and those structures osmotically equilibrated with the DVR. $-F_{2v}(x_2)[C_{2M}(x_2)-C_{2M}(x_1)]$

is the flow averaged change in concentration in the AHL, so is the solute added to DHL, CD, and AVR from the AHL; $T_W(x_2)$ is the solute entering the stage from deeper structures due to concentration difference between AVR and DVR; $T_W(x_1)$ is solute leaving the stage due to concentration difference between AVR and DVR; (The entering solute is potentially available for concentration within this stage and can be added to AHL solute. The leaving solute is wasted so far as concentration within the stage is concerned); and $F_{3v}(L) - F_{2v}(x_2)$ is equal to the sum of axial flux in DHL, CD, and vasa recta within the stage; thus, essentially, Eq. (9) states that the concentration change within the stage equals solute added, divided by the volume flow.

There are various ways equation (9) can be normalized. If we divide through by $C_{DVR}(x_2)$ and define $r(x_2,x_1) = C_{DVR}(x_2)/C_{DVR}(x_1)$ we obtain

$$1 - \frac{1}{r(x_2,x_1)} = \frac{-F_{2v}(x_2)[C_{2M}(x_2) - C_{2M}(x_1)] + T_W(x_2) - T_W(x_1)}{F_{3v}(L)C_{3M}(L) - F_{2v}(x_2)C_{2M}(x_2) + T_W(x_2)}. \quad (10)$$

This can be normalized to

$$r(x_2,x_1) = \frac{1}{1 - f_T(x_2,x_1)[1 - f_U(x_2,x_1)][1 - f_W(x_2,x_1)]} \quad (11)$$

where

$$f_T = \frac{-F_{2v}(x_2)[C_{2M}(x_2) - C_{2M}(x_1)] + T_W(x_2)}{- F_{2v}(x_2)C_{2M}(x_2) + T_W(x_2)} \quad (12)$$

$$f_U = \frac{F_{3v}(L)C_{3M}(L)}{F_{3v}(L)C_{3M}(L) - F_{2v}(x_2)C_{2M}(x_2) + T_W(x_2)} \quad (13)$$

$$f_W = \frac{T_W(x_1)}{- F_{2v}(x_2)[C_{2M}(x_2) - C_{2M}(x_1)] + T_W(x_2)}. \quad (14)$$

(2) Differential equations for mass balance in an n-stage single solute central core model of the renal medulla have been formulated. In this model we suppose that descending limbs of Henle (DHL) turn at discrete medullary levels to become ascending limbs of Henle (AHL). If N_j turn at the j'th level, we have

$$\sum_{j=1}^{\ell} N_j = N , \quad (15)$$

where N is the total number of loops. The volume flow in AHL that arise at the p'th level will be constant (because there is essentially no volume absorption from AHL). If we designate the axial volume flow in these AHL by $F_{2v,p} = -F_{1v,p}(x_p)$, then for each AHL we have the equation

$$F_{1v,p}(x_p)\frac{dC_{2s,p}(x)}{dx} = J_{2s,p}(x) \qquad \begin{matrix} j \leq p \leq \ell \\ x_{j-1} < x < x_j \end{matrix} \quad (16)$$

From an argument identical to that given above in the derivation of the mass balance equation, it follows that the combined flow in DHL, core and collecting ducts (CD) is given by

$$F_{vj} = F_{3v}(L) + \sum_{p=j}^{\ell} F_{1v,p}(x_1). \quad (17)$$

We then obtain the differential equation for osmotically equilibrated core structures

$$F_{vj} \frac{dC_4(x)}{dx} = \sum_{p=j}^{\ell} J_{2s,p}(x) \qquad x_{j-1} \leq x \leq x_j \tag{18}$$

If the $J_{2s,p}$ are Michaelis-Menten sources, these equations can be integrated. In the integrated equations the flows $F_{1v,p}(x_p)$ and $F_{3v}(L)$ appear as constants. These flows can be related to the entering flows and concentrations by the previously derived relation

$$\frac{F_{1v,p}(x_j)}{F_{1v,p}(x_{j-1})} = \left[\frac{C_{1p}(x_{j-1}) + A_{1p}C(0)}{C_{1p}(x_j) + A_{1p}C(0)}\right]^{1/\sigma_{1p}}, \tag{19}$$

with similar equations for collecting duct; in (19)

$$A_{1p} = \frac{N_{1s,p}}{L_{1p,p}\sigma_{1p}^2 \, RT \, C(0)}, \tag{20}$$

where $N_{1s,p}$ is solute permeability, $L_{1p,p}$ is hydraulic permeability, and σ_{1p} is the reflection coefficient.

In principle the above equations are sufficient to determine the unknown flows and concentrations, given entering flows, concentrations, membrane permeabilities, and reflection coefficients. In a few cases we can get explicit analytic solutions, but in general they must be solved numerically. To do this and to explore the parameter space of these models, we are using the continuation algorithm called SCOUT (described in project Z01 HL 03202-10 STB).

We have continued research on the qualitative analysis of the differential equations describing kidney models. The emphasis has been on the systems of equations that model diffusion and flow of water and solutes in a family of interchanging tubes. Work has continued on a boundary value problem for a counterflow system consisting of an arbitrary number of parallel flow tubes. In the model, some tubes are allowed to have positive diffusion and other tubes have zero diffusion. The system of differential equations is of the form

$$-DC_i'' + (F_iC_i)' + J_{si}(x, C_1, \ldots, C_m) = 0,$$

$$F_i' + J_{vi}(x, C_1, \ldots, C_m) = 0,$$

where C_i and F_i respectively denote the steady state solute

concentration and volume flow in the i-th tube, and where J_{si} and J_{vi} respectively denote the total transmembrane solute flux and volume flux out of tube i. The boundary conditions imposed are defined in terms of the geometry of the flow tubes. Existence theorems are given for solutions of these boundary value problems. These theorems are established by means of fixed point arguments and a priori bounds on the solutions. They require assumptions on the flux formulas that are frequently satisfied in cases of physiological interest. A paper [4] giving these results has been accepted for publication.

A detailed analyses of the existence and multiplicity of solutions of a 3-tube model of the renal medulla has been made. The tubes of the model represent the descending and ascending limb and the central core. While the equations of the model are of the form described above, the flux formulas include an active transport term that is not covered by the above results. For this 3-tube model, an existence theorem is obtained for solutions of the boundary value problem. By means of a heuristic, singular perturbation argument, it is demonstrated that the model admits multiple solutions. The geometry of the solution surface, and the stability of the solutions, are explored. It is shown that variations in the strength of the metabolic pump may cause the steady state solution of the model to undergo discontinuous jumps and to display the behavior of a hysteresis loop. A report describing these results is being written.

A report on earlier work on pressure driven flow in a single tube exchanging with an interstitium of known, non-constant concentration, has been recently published [3].

Proposed Course: It is planned to continue the analysis of two and three stage models. In particular, with the aid of roots finding algorithm it should be possible to make a rather detailed exploration of their parameter space. It is also planned to continue analytic investigation of two solute models and to incorporate an approximate analytic treatment of vascular washout into single solute models.

Publications:

1. Stephenson, John L. Case studies in renal and epithelial physiology. Lectures in Applied Math., Amer. Math. Soc. 19:171-212. (1981).

2. Stephenson, John L. Concentrating engines and the kidney. IV. Mass balance in a single stage of a multistage model of the renal medulla. Math Biosci. 54: (In press).

3. Garner, J. B., and Kellogg, R. B. The diffusion-convection equation with pressure. J. Math. Anal. and Applications. 79:58-70, (1981).

4. Garner, J. B. and Kellogg, R. B. Diffusion and convection in a family of tubes. J. Math. Anal. and Applications (In press).

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

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 03202-10 STB

PERIOD COVERED

October 1, 1980 through September 30, 1981

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
	R. Tewarson	Prof. SUNY, Stony Brook, L. I., New York	
	R. Kaimal	Visiting Fellow, Fogarty International Center	

COOPERATING UNITS (if any)

SUNY, Stony Brook, L.I., New York, and NIAMDD, Mathematical Research Branch

LAB/BRANCH

SECTION

Section on Theoretical Biophysics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.7	1.5	.2

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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 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.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: The purpose of this project is to develop a computer simulation of the mammalian kidney that gives a realistic description of function. This will permit the correlation of micropuncture and macroscopic clearance data with membrane transport characteristics.

Major Findings:

The existence of multiple steady states for certain models of the renal medulla has been recognized by us for some time. Methods for locating and classifying these states have been devised (R. Mejia, A Path Following Method for Obtaining a Connected Component of the Solution Surface of a Convection-Diffusion Model in Fluid Flow, abstract, Annual Fall Meeting of the Maryland-District of Columbia-Virginia section of the Mathematical Association of America, November 1980). A method for obtaining such solutions for a multinephron kidney model and for studying the transition from one state to another as a function of renal parameters has been described (R. Mejia and J. L. Stephenson, Multiple Solutions of Convection-Diffusion Equations Describing the Renal Concentrating Mechanism, Numerical Methods for Engineering, GAMNI 2, 2nd International Congress, 1003-1013, 1980). The main conclusions of these investigations are (1) multiple stable steady states appear to be physiologically plausible; (2) such states are connected by hysteresis loops that might stabilize operation in substantially different regimes; (3) some paths, e.g., as a function of certain model parameters, are forbidden. These simulations have suggested that appropriate experiments might give evidence of such multiple states.

An interactive continuation algorithem called SCOUT due to H-O. Peitgen, H. Jurgens and D. Saupe at Universität Bremen, has been implemented on the PDP-10 and PDP-11 computers at the NIH Computer Center. It consists of a set of simplicial continuation utilities that accept a simple, FORTRAN, description of the set of equations whose roots are sought. It will homotope to a root from an initial guess; it will continue as a function of a parameter; allows for a variable mesh size; and uses a perturbation technique to resolve bifurcation points or seek disjoint solution branches. SCOUT has been used to map the solution surface for some analytic models and has served to verify results obtained for some small (up to 15 finite difference equations) discretized models using methodology described below.

SCOUT, although at the forefront of the state-of-the-art in numerical continuation, cannot begin to handle the nonlinear systems with hundreds of unknowns that arise in multinephron kidney models. Hence, the (partitioned) algorithm used to solve the time dependent kidney equations for many nephrons (R. Mejia and J. L. Stephenson, Numerical Solutions of Multinephron Kidney

Equations, J. Comput. Phys. Vol 32, Number 2, 235-246, 1979) has been extended. An adaptive multigrid method for solution of a multipoint boundary value problem has been developed. The $O(h^2)$ approximation used to solve the tubular equations in the partitioned algorithm has been replaced by an $O(h^4)$ scheme (R. P. Tewarson, On the Use of Simpson's Rule in Renal Models, Math. Biosci., to appear). These have been solved symbolically (R. Mejia and J. L. Stephenson, Symbolics and Numerics of a Multinephron Kidney Model, 1979 MACSYMA User's Conference, Washington, D. C., 596-603. The interstitial variables and the boundary equations are discretized using a difference scheme with space mesh size of h/2, which is conservative. The partitioned system is then solved using an adaptive, alternating method (R. Mejia and J. L. Stephenson, An Alternating Method of Solution of a Multipoint Boundary Value Problem, SIAM 1979 Fall Meeting, Denver, Colorado). Tests indicate that the increased accuracy in conjunction with a coarser mesh will permit solution of models with several nephron populations, something that is required, for example, in the study of multisolute systems.

Proposed Course

(1) It is planned to incorporate a more detailed model of transmural transport into the proximal tubule in our whole kidney model. This model will be based on the models we have developed for transepithelial transport. One use of the extended model will be to study factors affecting urea cycling and excretion. We have begun a major effort to develop models which will make possible the analysis of renal washout curves following arterial injection of a variety of substances such as PAH and radioactive tracers. A major factor in determining the shape of these curves is the loading and unloading of the various cellular compartments in the kidney including the renal cells. This about doubles the size of the models, but our numerical methods of solving the models are now efficient enough so that we believe inclusion is possible.

A rational analysis of such washout curves should revitalize this area of renal physiology. Unlike micropuncture and isolated tubule experiments, this technique would be readily transferable to the study of the function of the human kidney in both health and disease.

Publications

Mejia, R. and Stephenson, J. L.: 1980. Multiple solutions of convection-diffusion equations describing the renal concentrating mechansism. Proc. International Congess of Numerical Methods for Engineering. GAMNI, DUNOD, Paris, France, pp. 1003-1013.

Tewarson, R. P. 1981. On the use of Simpson's rule in renal models. Math. Biosciences (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03203-06 STB

PERIOD COVERED
October 1, 1980 through September 30, 1981

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: A. Weinstein Research Fellow, Rogosin Kidney Center, Cornell Medical Center, New York, N. Y.

COOPERATING UNITS (if any)
Laboratory of Kidney and Electrolyte Metabolism, NHLBI, and NIAMDD, Mathematical Research Branch

LAB/BRANCH

SECTION
Section on Theoretical Biophysics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.8	.4	.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 the theory of solute and water transport across epithelia. To this end, mathematical models have been developed that permit computer simulation and approximate analytic treatment of electrolyte and non-electrolyte transport in a variety of steady state and timed experiments. Current work has focused on the nature of the coupling of solute-solute and solute-solvent fluxes and on the estimation of model parameters from experimental data.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives: The purpose of this project is to develop the theory of solute and water transport across epithelia. To this end, comprehensive numerical models have been developed that permit computer simulation of epithelial behavior in a variety of experimental settings. It is the aim of this work to utilize such models in the design of experiments as well as in the analysis of experimental data. Further, the numerical data from these models are used in the validation of approximations employed in analytical models of transport.

Work on this project during the past year has been directed primarily toward publication of completed work. All this work has now appeared or is in press.

Proposed Course: It is hoped to extend work on the electrolyte model to include the effect of bicarbonate in the bathing solutions.

Publications:

1. Weinstein, A. M., Stephenson, J. L., and Spring, K. R.: Chapter 12, The Coupled Transport of Water. in MEMBRANE TRANSPORT. "Comprehensive Biochemistry" series, edited by S. L. Bonting and J.J.H.H.M. de Pont (In press).

2. Weinstein, A. M. and Stephenson, J. L.: 1981. Models of coupled salt and water transport across leaky epithelia. J. Membrane Biol. 60:1-20.

3. Weinstein, A. M. and Stephenson, J. L.: Coupled water transport in standing gradient models of the lateral intercellular space. Biophysical J. (In press).

ANNUAL REPORT OF THE

CLINIC OF SURGERY

NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

October 1, 1980 through September 30, 1981

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.

Bioprosthetic Valvular Failure. The Fate of Bioprosthetic Valves in Young and Growing Animals. The development of an ideal cardiac valve substitute remains a major problem in cardiac surgery. Bioprosthetic cardiac valves have become the valves of choice at many institutions, primarily because they do not require chronic anticoagulant therapy. However, it has become apparent that the long-term durability of bioprosthetic valves is finite; degeneration and calcification of these valves have become major complications of long-term implantation.

The clinical, hemodynamic and morphologic findings were studied in 18 young sheep in which procine valvular bioprostheses (8 animals) and bovine pericardial bioprostheses (10 animals) were implanted in the tricuspid position. At the time of terminal elective studies (mean = 5.2 $\pm$ 0.2 months after implantation), 6 animals had ascites, 16 had hepatic congestion, and 4 had bioprosthetic valvular infection. Hemodynamic studies (n = 10) showed that the tricuspid transvalvular mean diastolic gradients were not different at implantation and at termination of the study (4.7 $\pm$ 0.8 vs. 4.9 $\pm$ 0.9 mm Hg); however, tricuspid valve end-diastolic gradients increased from 1.2 $\pm$ 0.6 mm Hg to 3.9 $\pm$ 0.5 mm Hg ($p < 0.01$). Each of the 18 valves had calcific deposits. Quantitative studies revealed that implanted porcine valvular bioprostheses (n = 7) contained a mean of 323 $\pm$ 165 mg of calcium/gm of dry weight of cuspal tissue, in contrast to 0.2 mg/gm in unimplanted porcine valvular bioprostheses. Similarly, implanted bovine pericardial bioprostheses (n = 6) contained a mean of 421 $\pm$ 115 mg of calcium/mg of dry weight of cuspal tissue, in contrast to 0.3 mg/gm in unimplanted bovine pericardial bioprostheses. Morphologic findings in both types of bioprostheses included calcific deposits, collagen degeneration, leaflet immobilization and retraction, and fibrous sheaths. The latter were more extensive in bovine pericardial bioprostheses than in porcine valvular bioprostheses. It was condluded: 1) that the pathological alterations which develop in bovine pericardial bioprostheses are generally similar to those in porcine valvular bioprostheses, but may be more severe; 2) that these alterations lead to physiological and clinical sequelae similar to those of bioprosthetic valvular failure in humans, and 3) that young sheep constitute an excellent experimental model for in vivo testing of bioprosthetic cardiac valves.

Late Results after Operations for Left Ventricular Outflow Tract Obstruction. We evaluated the late results of operations for the relief of left ventricular outflow tract obstruction in young patients, 1-18 years old, from our institution who have been followed for at least five years and from

studies in the recent literature which had average follow-up durations of five or more years. Operative mortalities for our series and those series we reviewed were low: 1.9% of 522 patients with valvular aortic stenosis, 6.0% of 222 patients with fixed subvalvular aortic stenosis and 5.5% of 18 patients with hypertrophic subaortic stenosis. From our series, gradients early postoperatively were decreased to less than 50 mm Hg in 88% (30/34) with valvular aortic stenosis, in 68% (15/22) with fixed subvalvular aortic stenosis and in 88% (8/9) with hypertrophic subaortic stenosis. Late survivals for patients in the combined series were: 90% (472/522) for valvular aortic stenosis, 86% (190/222) for subvalvular aortic stenosis and 82% (14/17) for hypertrophic subaortic stenosis, after mean follow-up periods of 5-14.4 years. All of our late survivors have had symptomatic improvement; 95% (58/61) are asymptomatic. However, for our patients actuarial analysis predicts that 50 $\pm$ 8% of those with valvular aortic stenosis and 44 $\pm$ 10% of those with subvalvular aortic stenosis after ten years will be free from the adverse postoperative events of residual and/or recurrent left ventricular outflow tract obstruction, clinically significant aortic regurgitation, reoperation, endocarditis or late death. Using the same adverse postoperative events to determine satisfactory late results from the combined series, we found that 54% (281/522) of those operated upon for valvular aortic stenosis, 54% (120/222) of those operated upon for subvalvular aortic stenosis and 78% (14/18) of those operated upon for hypertrophic subaortic stenosis had satisfactory late results 5-14 years after operation. Of our patients having unsatisfactiry late results, major hemodynamic abnormalities were detected in 55% (23/42) within one year postoperatively. Thus, it appears that operations for most children with left ventricular outflow tract obstruction are palliative ones. These patients should have early postoperative assessments and continuing long-term follow-up evaluations during childhood, adolescence and adulthood.

Identification of Human Cardiac Myosin Isoenzymes. A previous article from this laboratory reported no significant differences in the cardiac myosins of patients with obstructive hypertrophic cardiomyopathy and normals based on purified myosin ATP'ase activity and light chain composition by one-dimensional SDS-polyacrylamide gel electrophoresis. Since that report, several animal models of cardiac hypertrophy have shown the presence of myosin isoenzymes, due to variants in heavy chain structure. Hoh and associates, using a technique of pyrophosphate gel electrophoresis, demonstrated myosin isoenzymes in the rat which change in distribution with age and with the impaired contractile state of hypothyroidism. Flink and Morkin, in the rabbit, used 2-dimensional mapping of cyanogen bromide digests of myosin to demonstrate a new myosin isoenzyme in hypertrophied rabbits. Schwartz and her associates demonstrated a similar isoenzyme shift in the rat with hypertrophy induced by aortic banding. Lompre has demonstrated marked heavy chain isoenzyme changes in the newborn rat and rabbit hearts. In view of these recent developments in the study of cardiac myosin isoenzymes, myocardial tissue from patients with hypertrophic cardiomyopathy and from newborns was studied using techniques to evaluate heavy chain structural differences.

Human cardiac myosins were prepared from autopsy samples from 9 adults, 7 infants, and from surgical specimens from 7 patients undergoing left ventricular septal myectomy for obstructive hypertrophic cardiomyopathy. Infant myosin differed from adult by the presence of a 26,000 dalton fetal light chain, and depressed myosin ATPase's as activated by actin in 0.01 M KCl (64 $\pm$ 2 vs. 124 $\pm$ 2 nmoles P_i/mg/min at 37° C; $p < 0.05$) or by K^+-EDTA in

0.5M KCl (0.62 ± 0.07 vs. 1.21 ± 0.07 µmoles P_i/mg myosin/min at 37° C; $p < 0.001$). These myosins did not differ by pyrophosphate polyacrylamide gel electrophoresis, or proteolytic peptide mapping with alpha chymotrypsin, papain, or cyanogen bromide. Myosins from patients with obstructive hypertrophic cardiomyopathy did not differ from normals by enzymatic activity, pyrophosphate polyacrylamide gel electrophoresis, or peptide mapping. While these results fail to support a role for ventricular myosin heavy chain variation in the patients studied, they do confirm the existence of a human fetal light chain isoenzyme of cardiac myosin which differs from the adult in actin-activated MgATPase activity.

Evaluation of Operative Treatment for Discrete Subaortic Stenosis. Between 1956 and 1979 we performed complete resection of the fibrous ring in 53 patients with discrete subaortic stenosis. Six patients had associated anomalies: patent ductus arteriosus, ventricular septal defect, right ventricular outflow obstruction, and/or aortic to right ventricular fistula. Five patients suffered perioperative deaths; 4 were during our very early experience. Excluding 12 foreign patients, late follow-up evaluations including cardiac catheterizations were performed at our institution for all 36 survivors 1 to 24 years (mean 12.2 years) postoperatively. Gradients preoperatively averaged 98 mm Hg (35-190); gradients at 2 to 168 months (mean 18 months) postoperatively averaged 38 mm Hg (0-200). Eight patients had residual gradients over 50 mm Hg, of whom 6 had the tunnel form of left ventricular outflow obstruction. There were 6 late cardiac deaths. Of the long-term survivors 23 of 30 are symptomatic. Six patients have required reoperation, and 3 patients have developed bacterial endocarditis. Aortic regurgitation was present in 22 patients preoperatively; 5 additional patients have it late postoperatively. Cardiothoracic ratios averaged 0.53 preoperative and 0.44 late postoperative. Electrocardiographic criteria for left ventricular hypertrophy were present in 26 patients preoperative and in 10 late postoperative. Average echocardiographic LV systolic and diastolic dimensions were normal late postoperatively, 30 and 49 mm, respectively. Actuarial survival is 94% at 5 years (n = 28), 87% at 10 years (n = 22), and 81% at 20 years (n = 7). Survival without adverse cardiac abnormalities (reopration, bacterial endocarditis, residual gradient $>$ 50 mm Hg, cardiothoracic ratio $>$ than 0.60, or definite left ventricular hypertrophy by ECG criteria) is similar at 5 years (54%) and at 10 years (55%). We conclude that resection relieves symptoms and obstruction and gives satisfactory late results for most patients with discrete subaortic stenosis, but residual cardiac abnormalities require continuing long-term follow-up.

Mitral Valve Replacement with a Hancock Bioprosthesis - A Five to Ten Year Follow-up. In order to assess late results with the Hancock bioprosthesis, 111 patients undergoing mitral valve replacement, either along (56) or in conjunction with another type of prosthetic valve, prior to 1975 were evaluated.

Hancock bioprostheses were implanted in the mitral position in 111 patients prior to 1975. Isolated mitral valve replacement was performed in 56 patients, and the remaining 55 patients had either an additional bioprosthesis or mechanical prosthesis in another position. The hospital mortality was 9.9%. Cumulative follow-up is 505 patient years; mean 5.4 years. Late mortality for isolated mitral valve replacement is 4.3 ± 1.3% patient-years; actuarial survival is 82 ± 6% at five years and 65 ± 11% at ten years. Myocardial infarction and congestive failure were the most common causes of death following mitral valve replacement. Death secondary to intrinsic valve failure was seen

in one patient and prosthetic valve infection and dysfunction in one patient. Anticoagulant therapy was employed only for documented emboli or concomitant mechanical aortic prostheses. The incidence of emboli was 3.3 ± 0.9%/patient years for 62 patients with bioprostheses only. Emboli occurred in 22% of the patients in atrial fibrillation compared to 3.8% of patients in normal sinus rhythm. This would suggest that patients win chronic atrial fibrillation should receive anticoagulant therapy, preferably antiplatelet therapy rather than Warfarin to avoid the reported 5%/year anticoagulant complication rate.

Cardiac catheterization revealed good early hydraulic function of the Hancock bioprosthesis in the mitral position. As reported previously late hemodynamic data indicated progressive stenosis in 7 of 18 patients studied. Three of the seven patients were reoperated upon more than one year ago and the remaining four are being followed. The etiology of progressive prosthetic stenosis has been collagen degeneration and calcification in two patients and "stent creep" in one.

Valve failures have occurred in 10 patients (12 valves); one of these patients had a second failure 47 months after reoperation. Two tricuspid valves failed in these patients in addition to the ten mitrals. Nine of these patients were reoperated upon, with two deaths, and the tenth died before reoperation of infection. Actuarial late survival free of intrinsic mitral bioprosthetic failure was 99 ± 1% at five years, 92 ± 4% at seven years, 70 ± 12% at nine years, and 61 ± 13% at ten years. Modes of failure were gradual and were secondary to collagen degeneration, calcification, thrombosis and "stent creep."

Seven patients in our series have had documented sepsis with no prosthetic involvement requiring removal of the prosthesis or recurrence of infection once antibiotics were discontinued. One patient developed three episodes of endocarditis after triple valve replacement with Hancock bioprostheses; all three valves were destroyed when examined at necropsy 40 months after implantation. No postoperative Myobacterium chelonei infections have occurred in our series. Hemolysis has not been seen following atrioventricular replacements and perivalvular leaks are uncommon with the Hancock bioprosthesis.

It is unknown at the present time whether the long-term risk of late intrinsic valve failure and reoperation will outweigh the low incidence of emboli and avoidance of anticoagulant-related hemorrhage. Until further information becomes available, the Hancock bioprosthesis is used for mitral valve replacement only in patients older than 60 years or in patients with contraindications for anticoagulant therapy.

Hemodynamic Results Following Aortic Valve Replacement with the Hancock Standard Orifice Bioprosthesis. Since July 1974 approximately 120 standard orifice Hancock bioprostheses have been implanted in the aortic position. A concerted effort is made by most cardiac surgeons to implant the largest aortic prosthesis to avoid a significant resting or exercise gradient. An aortic annulus enlargement procedure must be performed to allow a larger prosthesis to be inserted in some patients which adds to the complexity and risk of the operation. In order to determine a need for this additional procuedre, postoperative cardiac catheterization data from 73 patients were reviewed. All patients underwent aortic valve replacement with Hancock standard orifice bioprostheses; annulus enlargement was not employed. Patients

returned to NIH six months postoperatively to undergo examination and cardiac catheterization. In addition to the standard catheterization, isoproterenol infusion was given to 15 patients to evaluate exercise gradients across the prostheses.

Postoperative cardiac catheterization was performed in 73 of these patients. Postoperatively, all but 2 patients were New York Heart Association Class I or II. Peak systolic gradient, cardiac index, body surface area, and effective valve orifice area were determined. Resting data, mean ± SEM, were:

Size	No	BSA (M^2)	PSG (mm Hg)	EOA (Cm^2)
21	15	1.63 ± 0.03*	10 ± 3+	1.27 ± 0.17#
23	25	1.83 ± 0.04	7 ± 2	1.46 ± 0.11
25	20	1.92 ± 0.04	7 ± 2	1.72 ± 0.20
27	13	1.92 ± 0.05	5 ± 3	1.97 ± 0.06

* = $p<0.001$ 21 vs 23,25,27 + = N.S., # = $p<0.05$ vs 25,27

Cardiac index (2.6 ± 0.1 L/min/M^2) and effective valve orifice area index (0.86 ± 0.05 Cm^2/M^2) were similar for all sizes.

Isoproterenol infusion (15 pts.) increased the peak systolic gradient from a mean 10 mm Hg to a mean of 44 mm Hg (range 10-85) independent of valve size. Residual peak systolic gradient or small effective valve orifice area did not preclude satisfactory clinical improvement. The small peak systolic gradient found in valve sizes 21 and 23 are satisfactory for patients with small body surface areas and, therefore, we conclude annulus enlargement is rarely necessary.

Mitral Valve Replacement after Closed Mitral Commissurotomy. Closed mitral commissurotomy was performed on 232 patients (67M, 165F) with isolated mitral valve disease from 1954-1980. Mean age was 41 years and operative mortality was 4%. Pre- and postoperative catheterization was obtained in 70% of patients. Mean New York Heart Association Functional Class preop was 2.7 and improved to 1.6 after closed mitral commissurotomy. Mean mitral valve gradient decreased from 15 ± 0.5 to 6 ± 0.4 ($p<.001$). Functional Class was 1.6 and 1.8 at 5 and 10 years after closed mitral commissurotomy; 20 patients (10%) underwent more than one closed mitral commissurotomy. Actuarial survival free of mitral valve replacement was 83 ± 3% and 66 ± 4% at 5 and 10 years. Mitral valve replacement was required in 42 patients (15M, 27F) a mean of 9 years after closed mitral commissurotomy (range 1-26 years). In this subgroup the closed mitral commissurotomy initially improved Functional Class from 2.7 to 1.9 and mean mitral valve gradient from 13 ± 0.8 to 6 ± 0.9 ($p<.001$). Patients subsequently undergoing mitral valve replacement had a higher mean LA pressure after closed mitral commissurotomy than those who did not require mitral valve replacement (18 ± 1.2 vs 13.5 ± 0.6, $p<.001$). Operative mortality for mitral valve replacement was 12%. Mitral valve replacement improved Functional Class from 3.1 to 1.7 and mean mitral valve gradient from 12 ± 1.1 to 5 ± 0.6 ($p<.001$). At both 5 and 10 years after mitral valve replacement Functional Class was 2.0. Closed mitral commissurotomy provides useful palliation even in 20% of patients who ultimately require mitral valve replacement.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02684-03 SU

PERIOD COVERED October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Diastolic retroperfusion of acutely ischemic myocardium utilizing a balloon tipped coronary vein catheter

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

PI: Steven R. Gundry, M.D., Clinical Associate, Clinic of Surgery, NHLBI

OTHER:

Daniel M. Goldfaden, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Michael Jones, M.D., Senior Surgeon, Clinic of Surgery, NHLBI
Harry W. Seipp, Chief Technician, Clinic of Surgery, NHLBI

Rodger E. Solomon, Electrical Engineer, DRS

COOPERATING UNITS (if any)

Biomedical Engineering, DRS

LAB/BRANCH
Surgery

SECTION

INSTITUTE AND LOCATION
NHLBI-NIH, Bethesda, MD. 20205

TOTAL MANYEARS: 3	PROFESSIONAL: 3	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)

Despite advances in intra-aortic balloon pumping and emergency myocardial revascularization, salvage of ischemic myocardium has been limited in man by the lack of effective collateral circulation into an area of acute infarction. We are currently carrying out experimental trials of perfusing ischemic myocardium by retrograde diastolic pulsation of oxygenated blood into the coronary veins draining an area of ischemia via a balloon tipped catheter that can be introduced transvenously. Results to date indicate correction of EKG changes, reversal of dyskinetic areas and improvement in myocardial performance in acutely ischemic myocardium following institution of retroperfusion.

PHS-6040
(Rev. 2-81)

Description: A double lumen balloon-tipped catheter is placed retrogradely into the coronary sinus in an experimental animal and advanced into the left anterior descending coronary vein to an extent that when the balloon is partially inflated the lumen is obstructed, but unobstructed to normal systolic venous flow when deflated.

This catheter is connected to an oxygenated blood source, the brachial artery, through a synchronized 20 cc. pulsatile assist device which is driven by a standard electrocardiogram-actuated intraaortic balloon counterpulsation pump. After creating an area of ischemic myocardium by LAD ligation, and recording myocardial dynamics with flow transducers and intramyocardial ultrasonography, the coronary vein perfusion pump is actuated. Oxygenated blood is injected retrograde into the coronary vein in diastole via the balloon catheter, whose balloon simultaneously fills and obstructs venous drainage during the injection. The pump is then deactivated during systole. The balloon deflates, and blood drains from the coronary vein into the right atrium in the normal fashion. Myocardial dynamics are reassessed during this procedure.

We designed and experimentally evaluated a transvenously introduced double-lumen balloon tipped catheter and ECG activated pumping system for perfusing ischemic myocardium by retrograde pulsation of oxygenated blood into the coronary veins during diastole. Balloon deflation during systole allowed normal venous drainage. Sixteen dogs were instrumented with sonomicrometry crystals and catheters to measure regional and global left ventricular (LV) function. The left anterior descending coronary artery (LAD) was occluded for 40 minutes. Following 10 minutes of ischemia, the affected LV regions were dilated and dyskinetic. Coronary vein retroperfusion (CVRP) was instituted for 30 minutes in 8 dogs (controls were not perfused). CVRP restored 37% of LV systolic shortening (1.5 mm. vs 0.6 mm)*; controls had no systolic shortening. Regional LV dilation of 2.2 mm. was reduced to 1.5 mm. by CVRP; controls expanded to 3.1 mm.* Endocardial ST segments returned to normal with CVRP, but elevated 19 mm. in controls.* Cardiac output rose to baseline levels with CVRP but remained 1.5 L/min depressed in controls.* Three of 8 controls died. Four of 5 remaining dogs died from LV fibrillation after LAD release; all CVRP dogs recovered. CVRP offers a transvenous approach for assisting and perfusing failing, ischemic myocardium.* ($p<0.001$)

Proposed Course: The study has been completed.

Presentations:

Gundry SR: Diastolic retroperfusion of acutely ischemic myocardium utilizing a balloon tipped coronary vein catheter. 53rd Scientific sessions, American Heart Association, Miami, November, 1980.

Gundry, S.R.: Modification of myocardial ischemia in normal and hypertrophied hearts utilizing diastolic retroperfusion of the coronary veins. Presented at Samson Surgical Society, June 1981 - to be published in J. Thoracic Cardiovasc. Surg.

Publications: Gundry SR, Goldfaden DM, Seipp HW, Solomon RE, Jones M: Diastolic retroperfusion of acutely ischemic myocardium utilizing a balloon tipped coronary vein catheter. Circulation 62 (Suppl III): III-316, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02686 -03 SU

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Endothelial changes in human saphenous veins prepared for coronary artery bypass grafts: Effects of distention pressure and preservation techniques.

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

PI: Steven R. Gundry, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Michael Jones, M.D., Senior Surgeon, Clinic of Surgery, NHLBI

OTHER: Victor Ferrans, M.D., PhD., Chief, Ultrastructure sec., Pathology Branch
Tokuhiro Ishihara, M.D., Guest Investigator, Ultrastructure sec., Pathology Branch

COOPERATING UNITS (if any)

Pathology Branch, NHLBI

LAB/BRANCH

Clinic of Surgery

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2-1/4	2-1/4	

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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)

Endothelial changes in saphenous veins harvested for coronary artery bypass grafts were found when veins prepared in this manner were examined using the scanning and transmission electron microscopes. Using current distention pressures and preservation techniques, severe endothelial disruption was found. We compared the endothelial changes caused by a variety of currently employed vein handling techniques to determine the best method to protect venous endothelium during the bypass procedure.

PHS-6040
(Rev. 2-81)

DESCRIPTION: The saphenous vein has become the conduit of choice in coronary artery revascularization procedures. Despite its popularity and usefulness, clinical and pathological studies indicate that these vein grafts are subject to early closure and mild to severe intimal hyperplasia or atherosclerosis. Experimental models have demonstrated that endothelial damage or disruption can predispose to these conditions. To evaluate the endothelium of saphenous veins prepared in the usual manner at this Institute and others, a portion of the harvested vein taken for coronary artery bypass grafts in patients was immediately fixed in a distended condition at mean arterial pressure for electron microscopy. These veins were compared to veins which were distended and fixed at venous pressure alone.

Endothelial damage occurring during preparation of saphenous vein grafts is an important cause of early and late graft failure. To determine optimal preparation techniques for human saphenous veins (SV) we made a scanning electron microscopic comparison of the effects of handling techniques, immersion media and distention pressures on human SV morphology. Segments of SV from 30 patients were divided into 7 groups of 5 veins each. Group 1 was immediately distended and fixed with glutaraldehyde at 100 mm Hg. Groups 2 and 3 were immersed in normal saline for one hour at $4^{\circ}C$ and $28^{\circ}C$, respectively, and then distended to 100 mm Hg with saline. Groups 4 and 5 were immersed in blood for one hour at $4^{\circ}C$ and $28^{\circ}C$, respectively, followed by distention to 100 mm Hg with blood. Groups 6 and 7 were immediately distended with saline group (6) or blood group (7) to 300 mm Hg. One SV from each group was grasped with a vascular clamp; one SV in each group also contained a side branch which had been ligated. All groups were fixed by perfusion with glutaraldehyde at 100 mm Hg to simulate arterial pressure and examined with a scanning electron microscope. SV immersed in warm saline sustained massive endothelial cell loss, while SV immersed in warm blood showed only moderate damage. Cold blood and cold saline immersion fully preserved endothelium; however, saline immersion produced mural edema. Distention to 300 mm Hg with saline produced severe endothelial damage and edema, an effect lessened by blood distention. Vascular clamping destroyed endothelium and fractured the intima. Marked luminal stenoses were caused by 4 of 7 side branch ties that appeared normal externally. We conclude that human SV are best preserved by a "no touch" harvesting technique, minimizing manipulation, placement of side branch ties away from the SV wall, immersion in cold blood, and avoidance of distention above 100 mm Hg.

RESULTS: The project has been completed.

PUBLICATIONS: Gundry SR, Jones M, Ishihara T, Ferrans VJ: Optimal preparation of human saphenous vein grafts. Surgery 88: 785-794, 1980.

Jones M, Gundry SR, Ishihara T, Ferrans VJ: Reply to letter to the Editor: Intraoperative trauma to human saphenous veins: Scanning electron microscopic comparison of preparation techniques. Ann Thorac Surg (In Press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02696-02 SU

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Morphometric analyses of myocardial hypertrophy (compensated and decompensated) utilizing computer-assisted microdensitometric techniques

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

PI: Michael Jones, M.D., Senior Surgeon, Clinic of Surgery, NHLBI
J. B. Zwischenberger, M.D., Clinical Associate, Clinic of Surgery, NHLBI

Other: John J. Schief, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Benes L. Trus, Ph.D., Research Chemist, CSL, DCRT
James M. DeLeo, Computer Systems Analyst, CSL, DCRT
Victor J. Ferrans, M.D., Ph.D., Chief, Ultrastructural Sec. Path. Br., /NHLBI
William C. Roberts, M.D, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

Division of Computer Research & Technology and Pathology Branch, NHLBI

LAB/BRANCH
Clinic of Surgery

SECTION

INSTITUTE AND LOCATION
National Heart, Lung & Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2-1/2	2	1/2

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

☐ (a1) MINORS   ☐ (a2) INTERVIEWS

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

We have developed computer-assisted interactive techniques using mocrodensitometry and surface display facilities to perform micromorphometric analyses of hypertrophied myocardium from humans and from experimental animal preparations. Our techniques have the ability to analyze approximately 202,500 samples (pixels) from a photomicrograph of a tissue specimen. The size of the sample is determined by the magnification of the photomicrograph. Depending upon the information required, magnifications vary from 10 to 860 X, so that resolutions may be varied from 5 to 0.5 millimicrons. Techniques developed determine percent interstitium, cardiac muscle cell diameter and size, and ratios of cardiac muscle area to numbers of nuclei and capillaries with a precision not afforded by other techniques.

PHS-6040
(Rev. 2-81)

DESCRIPTION: Myocardial tissues which we have obtained for investigation include the following: 1) human cardiac muscle from patients operated upon because of right ventricular outflow tract obstruction, 2) left ventricular myocardium from Newfoundland dogs with naturally occurring, genetically transmitted, subaortic stenosis, 3) hypertrophied right ventricular myocardium from swine having had pulmonary artery constrictions, and 4) left and right ventricular myocardium for which the stimulus for hypertrophy has been produced *in utero* in fetal lambs.

The computer programs and packages have been developed for the micro-morphometric determinations of cardiac muscle cell diameters and cross sectional profile area, percentage of interstitium, and ratios of cardiac muscle cell area to numbers of nuclei and to numbers of capillaries. This portion of our investigation has been initiated upon animal tissues.

PROPOSED COURSE: A manuscript on the technique for computer assisted quantitative light microscopic analysis of myocardial tissue components is in preparation. This technique is currently being used to analyze the above described tissues.

We anticipate extending these techniques to three dimensional image analysis of normal and abnormal cardiac muscle cells. This investigation will involve reconstruction of cardiac muscle cells from thin (one millimicrometer) thick serial sections of human and animal cardiac muscle cells.

The data obtained from the micromorphometric analyses will be integrated with studies of clinical evaluations and with studies of ventricular function. Thus we will continue to investigate the interrelationships of myocardial morphology with compensated and decompensated myocardial hypertrophy.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02697-02 SU

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Characterization of naturally occurring, genetically transmitted, fibrous subaortic stenosis in Newfoundland dogs.

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

PI: Michael Jones, M.D., Senior Surgeon & Investigator, Clinic of Surgery, NHLBI

OTHER: A. Michael Borkon, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Victor J. Ferrans, M.D., Ph.D., Chief Ultrastructure Sec. Pathology Br.
William C. Roberts, M.D., Chief, Pathology Branch, NHLBI
Joseph E. Pierce, DVM, Chief, Sec. Laboratory Animal Medicine & Surgery, NHLBI

COOPERATING UNITS (if any)
Pathology Branch and Section of Lab Animal Medicine & Surgery, NHLBI

LAB/BRANCH
Clinic of Surgery

SECTION

INSTITUTE AND LOCATION
National Heart, Lung & Blood Institute

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3	2	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

We have been characterizing the morphologic and the hemodynamic abnormalities occurring in Newfoundland dogs with genetically transmitted subaortic stenosis. Of 130 animals studied thus far, almost one-half have hemodynamic and/or morphologic evidence for left ventricular outflow tract obstruction. The obstruction is absent at birth, appearing after one month of age. The lesion presents clinically by the presence of a precordial murmur, thrill, arrhythmia, congestive heart failure, bacterial endocarditis of the aortic valve, or sudden death. Obstruction to left ventricular outflow is caused by the development of a circumferential, subaortic fibromuscular ring continuous with the anterior leaflet of the mitral valve, virtually identical to the same lesion in humans. The obstruction is associated with left ventricular hypertrophy, intramural coronary artery lesions, myocardial fibrosis, and abnormalities of myocardial blood flow.

PHS-6040
(Rev. 2-81)

Description: In the 130 dogs studied hemodynamic and/or morphologic evidence of LV outflow tract obstruction (LVOTO) was present in 63 animals, including 34% of 53 < 1 year old, 50% of 47 aged 13 - 24 months, 74% of 23 aged 25 - 48 months, and 71% of 7 aged > 48 months. None of 22 newborns and 1 of 5 < 1 month old had LVOTO. No dogs had asymmetrical septal hypertrophy or myocardial cellular disarray. LVOTO was associated with LV hypertrophy (LV/body weights > 4.5 g/kg), sudden death (n=16), thickened aortic valves, bacterial endocarditis (n=8), intramural coronary artery lesions, myocardial fibrosis and abnormalities of myocardial blood flow. All dogs with LVOTO had circumferential subvalvular fibrous rings continuous with the ventricular septum and anterior mitral leaflet. Of 64 dogs without LVOTO the septal endocardium was thickened in 38%. Although present in a few dogs without LVOTO, an unusual angulation of the aorta and the septum occurred in dogs with LVOTO. Subaortic stenosis and its sequelae in Newfoundland dogs appears to be acquired after birth and its pathogenesis to be due to the interaction of genetic, morphologic, and hemodynamic factors.

Presentations:

Michael Jones, M.D.: Morphological-clinical correlates of myocardial fibrosis due to discrete subaortic stenosis in Newfoundland dogs. 30th Annual Scientific Sessions. American College of Cardiology. March, 1981.

Publications:

Jones M, Ferrans VJ, Roberts WC, Borkon AM, Pierce JE: Morphological-clinical correlates of myocardial fibrosis due to discrete subaortic stenosis in Newfoundland dogs. Am J Cardiol 47: 447, 1981.

Jones M, Ferrans VJ, Roberts WC, Borkon AM, Pierce JE: Congenital Heart Disease Acquired after Birth: Subaortic Stenosis in Newfoundland Dogs. Abstract submitted for 54th Scientific Sessions of the American Heart Association.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02698-02 SU

PERIOD COVERED October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Postoperative cardiac care of infants and children

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

PI: Michael Jones, M. D., Senior Surgeon & Investigator, Clinic of Surgery, NHLBI
Lily Ng, R.N., M.S.N., Supervisor Clinical Nurse, Clinical Center
Altagracia M. Chavez, M.D., Clinic Associate, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

Nursing Department, The Clinical Center

LAB/BRANCH
Clinic of Surgery

SECTION

INSTITUTE AND LOCATION
National Heart, Lung & Blood Institute

TOTAL MANYEARS: 3/4	PROFESSIONAL: 3/4	OTHER:

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[X] (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Cardiac surgery in infants and children has progressed so that most lesions may be corrected early in life. In theory and in practice the postoperative management of infants and children is similar to that of adult patients. Nevertheless, the potential for growth, the particular and often immature metabolic prosesses, and the cardiovascular physiology of congenital malformations demand special postoperative considerations. From our experiences in the Clinic of Surgery of the NHLBI and those in the Hospital for Sick Children, London, England, we have developed guidelines for postoperative management to assist nurses and clinical associates with care of young patients after cardiac operations.

PHS-6040
(Rev. 2-81)

DESCRIPTION: The special problems of postoperative cardiac care in infants and children have been divided into the following categories: 1) preoperative considerations; 2) choice of operative procedure; 3) initial postoperative assessment; 4) basic procedures; 5) cardiovascular problems, including heart rate, preload, afterload, contractility, subacute and chronic heart failure, and rhythm disturbances; 6) respiratory problems, including care of infants and children on ventilators, weaning from ventilatory support, and problems of phrenic nerve paralysis; 7) cardiopulmonary resuscitation; 8) fluid, electrolyte, and other metabolic problems; 9) nutrition; 10) body temperature regulation; 11) renal problems, including peritoneal dialysis; 12) hematological problems; 13) jaundice; 14) infection; 15) neurological problems; 16) medication dosages; 17) prognoses without and following operation; and, 18) parental counseling.

From the above delineated considerations we have developed postoperative management guidelines, particularly directed toward the initiate and limitedly experienced nurse and clinical associate assisting with the postoperative care of pediatric cardiac surgical patients.

PROPOSED COURSE: The postoperative management protocols for our unit are in the process of being refined. A publication specifically concentrating upon nursing considerations is in preparation.

Publications:

Jones, M. and Stark, J.: Special problems of postoperative care in infancy and childhood. In: Postoperative Cardiac Care. (Brainbridge, M.V., Ed) Blackwell Scientific Publications, Oxford, England. IN PRESS.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02699-02 SU

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Evaluation of operative treatment for discrete subaortic stenosis

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

PI: Glenn R. Barnhart, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Michael Jones, M.D., Senior Surgeon, Clinic of Surgery, NHLBI

Other: Andrew G. Morrow, M.D., Chief, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Clinic of Surgery

SECTION

INSTITUTE AND LOCATION
National Heart, Lung & Blood Institute

TOTAL MANYEARS: 1	PROFESSIONAL: 1	OTHER:

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Fifty-seven patients have undergone operation for discrete subaortic stenosis. There were six (10.5%) early deaths. Forty-two of these patients have been followed for up to 25 years (mean 12.8) after operation. Of 32 patients undergoing early postoperative cardiac catheterizations, 75% had satisfactory relief of obstruction. Of the 32 late survivors 69% are asymptomatic. Acturarial survival is 77 ± 7% at ten years. However, late survival without suffering the postoperative adverse events of residual obstruction, significant aortic regurgitation, endocarditis, complete heart block or reoperation is 39 ± 8% at ten years.

PHS-6040
(Rev. 2-81)

DESCRIPTION: Between 1956 and 1979 we performed complete resection of the fibrous ring in 53 patients with discrete subaortic stenosis. Six patients had associated anomalies: PDA, VSD, RV outflow obstruction, and/or Ao to RV fistula. Five patients suffered perioperative deaths; 4 were during our very early experience. Excluding 12 foreign patients, late follow-up evaluations including cardiac catheterizations were performed at our institution for all 36 survivors 1 to 24 years (mean 12.2 years) postoperatively. Gradients preoperatively averaged 98 mm Hg (35-190); gradients at 2 to 168 months (mean 18 months) postop averaged 38 mm Hg (0-200). Eight patients had residual gradients over 50 mm Hg, of whom 6 had the tunnel form of LV outflow obstruction. There were 6 late cardiac deaths. Of the long-term survivors 23 of 30 are asymptomatic. Six patients have required reoperation and 3 patients have developed bacterial endocarditis. Aortic regurgitation was present in 22 patients preop; 5 additional patients have it late postop. Cardiothoracic ratios averaged 0.53 preop and 0.44 late postop. ECG criteria for LVH were present in 26 patients preop and in 10 late postop. Average echocardiographic LV systolic and diastolic dimensions were normal late postop, 30 and 49 mm, respectively. Acturarial survival is 94% at 5 years (n=28), 87% at 10 years (n=22), and 81% at 20 years (n=7). Survival without adverse cardiac abnormalities (reoperation, bacterial endocarditis, residual gradient > 50 mm Hg, cardiothoracic ratio ≥ than 0.60, or definite LVH by ECG criteria) is similar at 5 years (54%) and at 10 years (55%). We conclude that resection relieves symptoms and obstruction and gives satisfactory late results for most patients with discrete subaortic stenosis, but residual cardiac abnormalities require continuing long-term follow up.

PROPOSED COURSE: The patients included in this report and additional patients as they undergo operation will be included in our continuing evaluation of the natural history of this operatively treated congenital cardiac anomaly.

Presentations:

Glenn R. Barnhart: Late results of operation for discrete subaortic stenosis. 30th Annual Scientific Sessions. American College of Cardiology. San Francisco, March 1981.

Publications:

Barnhart, G.R., Jones, M., and Morrow, A. G.: Late results of operation for discrete subaortic stenosis. Amer. J. Cardiol. 47:432, 1981.

Barnhart, G.R., Jones, M., and Morrow, A.G.: Prognosis of patients after operation for fibrous subvalvular aortic stenosis. Submitted for publication.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02702-02 SU

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Identification of human cardiac myosin isoenzymes

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

PI: John J. Schier, M.D., Clinical Associate, Clinic of Surgery, NHLBI

Other: Robert S. Adelstein, M.D., Director, Laboratory of Molecular Cardiology, NHLBI

Andrew G. Morrow, M.D., Chief, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

Laboratory of Molecular Cardiology, NHLBI

LAB/BRANCH
Clinic of Surgery

SECTION

INSTITUTE AND LOCATION
National Heart, Lung & Blood Institute

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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 previous article from this laboratory reported no significant differences in the cardiac myosins of patients with obstructive hypertrophic cardiomyopathy and normals based on purified myosin ATP'ase activity and light chain composition by one-dimensional SDS-polyacrylamide gel electrophoresis. Since that report, several animal models of cardiac hypertrophy have shown the presence of myosin isoenzymes, due to variants in heavy chain structure. Hoh and associates, using a technique of pyrophosphate gel electrophoresis, demonstrated myosin isoenzymes in the rat which change in distribution with age and with the impaired contractile state of hypothroidism. Flink and Morkin, in the rabbit, used 2-dimensional mapping of cyanogen bromide digests of myosin to demonstrate a new myosin isoenzyme in hypertrophied rabbits. Schwartz and her associates demonstrated a similar isoenzyme shift in the rat with hypertrophy induced by aortic banding. Lompre has demonstrated marked heavy chain isoenzyme changes in the newborn rat and rabbit hearts. In view of these recent developments in the study of cardiac myosin isoenzymes, myocardial tissue from patients with hypertrophic cardiomyopathy and from newborns was studied using techniques to evaluate heavy chain structural differences.

PHS-6040
(Rev. 2-81)

DESCRIPTION: Human cardiac myosins were prepared from autopsy samples from 9 adults, 7 infants, and from surgical specimens from 7 patients undergoing left ventricular septal myectomy for obstructive hypertrophic cardiomyopathy (HOCM). Infant myosin differed from adult by the presence of a 26,000 dalton fetal light chain, and depressed myosin ATPase's as activated by actin in 0.01M KCl (64 ± 2 vs 124 ± 2 nmoles P_i/mg/min at 37° C; $p < 0.05$) or by K+ -EDTA in 0.5M KCl (0.62 ± 0.07 vs. 1.21 ± 0.07 µmoles P_i/mg myosin/min at 37°C; $p < 0.001$). These myosins did not differ by pyrophosphate polyacrylamide gel electrophoresis, or proteolytic peptide mapping with alpha chymotrypsin, papain, or cyanogen bromide. Myosins from patients with HOCM did not differ from normals by enzymatic activity, pryophosphate polyacrylamide gel electrophoresis, or peptide mapping. While these results fail to support a role for ventricular myosin heavy chain variation in the patients studied, they do confirm the existence of a human fetal light chain isoenzyme of cardiac myosin which differs from the adult in actin-activated MgATPase activity.

Proprose Coures: The manuscript has been submitted for publication.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02705-02 SU

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Left Ventricular Hypertrophy Produced in Utero

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

P.I. Joseph B. Zwischenberger, M.D., Clinical Associate, Clinic of Surgery, NHLBI

Michael Jones, M.D., Senior Surgeon, Clinic of Surgery, NHLBI

OTHER: Delwin K. Buckhold, D.V.M., Lab. Animal Medicine and Surgery, NHLBI
John J. Schier, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Leland G. Siwek, M.D., Clinical Associate, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)
Section of Lab Animal Medicine and Surgery, NHLBI

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Clinic of Surgery

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National Heart, Lung, and Blood Institute

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Post ductal aortic coarctation (COA) was produced in utero in fetal lambs at 85 days gestation (term =147 days). Eight COA lambs were studied at age 10 weeks and 7 COA lambs were studied at age 20 weeks with 23 control animals of comparable age and weights. To evaluate left ventricular hypertrophy and the chronic effects of the experiment, the following studies were performed: (1) Gross morphometrics of the heart, lungs and liver. (2) Cardiac catheterization with transducer tipped catheters. (3) High quality left ventricular posterior wall echos. (4) Thick walled sphere stress-strain analysis of the left ventricle. (5) Hydroxyprolene content. (6) Cardiac light microscopy for interstitial fibrosis.

PHS-6040
(Rev. 2-81)

Description: Lamb fetuses at 85 of 145 days gestation had 5.5 mm. Teflon bands placed around the postductal thoracic aorta. Approximately 30 percent of banded fetuses survived to study. A mean gradient of 24.2 + 1.9 mmhs. (sem) resulted from an area stenosis of 78+2% produced by the banding. The following data represent least squares mean values and their standard errors calculated from linear regressions at the mean age and weight.

The COA group showed significant hypertension (165+6/139+5 (COA) vs. 126+3/106+3 (C) mmhg. $P < .001$) which increased in severity with age. The COA group also showed significant left ventricular hypertrophy (LV free wall/body wt. 2.0 + 0.1 (COA) vs. 1.5 + 0.1 (C) $P < .001$) with no evidence of congestive heart failure. The hypertrophy was not fully compensatory since the cardiac output normalized to body weight was depressed. In the COA group (92+4 (COA) vs. 110+3 (C) ml/min/Kg $P < .01$) and the stroke work normalized for LV weight was also depressed (0.036+.002 (COA) vs. 0.044+.002 (C) gm/g at both 10 and 20 weeks of age in the COA group.

Mechanical volume compliance, myocardial stiffness constant and hydroxyprolene content in the left ventricle of the excised potassium arrested hearts showed no significant difference between groups. Preliminary histologic studies suggest no difference in left ventricular fibrosis. This fetal model of COA is a source of continued studies regarding the mechanisms of cardiac compensation to chronic afterload produced in utero.

Proposed Course: Analysis of the data collected to date is in progress. Further studies of the tissue specimens from this model include myocardial micromorphometrics using computer assisted microdensitometry evaluation of the pulmonary microvasculature, and examination of the aortic wall changes secondary to the coarctation. Upon completion of the outlined studies, the pathophysiology of COA should be better understood.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02707-02 SU

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Reperfusion with blood cardioplegia provides improved recovery of both early and late ventricular function after ischemic injury

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

PI: Daniel M. Rose, M.D., Guest Worker, Clinic of Surgery, NHLBI

Other: Glenn R. Barnhart, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Jean-Paul Koch, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Micheal Jones, M.D., Senior Surgeon & Investigator, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

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INSTITUTE AND LOCATION
National Heart, Lung & Blood Institute

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

It has been proposed that modification of the initial reperfusion solution after aortic occlusion, may alter the extent of ischemic injury. We, therefore, studied initial reperfusion with either cold blood or cold blood cardioplegia after ischemic injury. Reperfusion with cold blood cardioplegia provides better recovery of ventricular function and compliance immediately, 21, and 120 days postoperatively than cold blood alone.

PHS-6040
(Rev. 2-81)

DESCRIPTION AND RESULTS: We studied initial reperfusion after aortic occlusion in 15 dogs divided into three equal groups. Group I underwent 2 hours of normothermic cardiopulmonary bypass (CPB); Group II underwent 2 hours of CPB including one hour of 20°C ischemic arrest; Group III was identical to Group II but initially was reperfused with 250 cc of 25°C blood cardioplegia (BCP, K^+ = 30 mEq/L) prior to aortic unclamping. Stroke work index (SWI), LVEDP, and Vpm were compared preoperatively, immediately postoperatively, at 21 days and 120 days postoperatively. Results of SWI (GmN) are summarized below ± SEM:

	Preop	Postop	21d	120d
Gp I	36.6 ± 4.7	30.6 ± 4.2	34.8 ± 4.2	30.1 ± 2.2
Gp II	41.9 ± 3.8	16.4 ± 3.3*	28.9 ± 4.1*	27.9 ± 3.7*
Gp III	37.0 ± 2.9	22.9 ± 3.5*	31.5 ± 2.5	30.1 ± 2.3

Both Gps II and III had significant* elevations in all postoperative values for LVEDP while there were no significant* differences in the values for Vpm between Gps II and III. Hearts which were initially reperfused with BCP (Gp III) had significantly* better recovery of SWI immediately postoperatively (62% vs 39%), at 21d (85% vs 69%), and at 120 d (81% vs 66%) than Gp II. We conclude that after ischemic injury, initial reperfusion with BCP provides better recovery of both early and late ventricular function than reperfusion with oxygenated blood alone.

*$p < 0.05$

COURSE: The project has been completed.

PRESENTATIONS:

Rose, D.M.: Reperfusion with blood cardioplegia provides prolonged recovery from ischemic injury. 42nd Annual Meeting, Society of University Surgeons, February 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02708-02 SU

PERIOD COVERED October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Long-term results of repair of incomplete persistent atriovantricular canal

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

PI: Daniel M. Goldfaden, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Michael Jones, M.D., Senior Surgeon, Clinic of Surgery, NHLBI
Andrew G. Morrow, M.D., Chief, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Late clinical evaulation was performed on 39 patients who had repair of incomplete persistent atrioventricular canal at the NIH prior to 1976. Average follow up duration is 13 (range 5-24) years. Twenty-six patients are asymptomatic at their most recent evaluation. Actuarial survival is 88 ± 6% at 13 years after operation; survival without reoperation is 82 ± 6%. However, survival free of any late complications, including late death, reoperation, arrhythmia, or symptomatic mitral regurgitation is 52 ± 10% at 13 years.

PHS-6040
(Rev. 2-81)

DESCRIPTION: We evaluated the late results following repair of otherwise anatomically uncomplicated persistent atrioventricular canal in 39 consecutive operative survivors who underwent operation at our institution prior to 1976. Average follow-up was 12 years. Postoperative cardiac catheterization was performed in 35 patients (90%) at an average of 11 months after operation. Seven (20%) had residual mitral regurgitation with elevated mean pulmonary arterial wedge or left atrial pressures with abnormal v waves. Regurgitation was mild to moderate (PAW or LA 12-15 mm Hg) in five and severe (PAW over 20 mm Hg) in two patients. Clinically significant arrhythmias including complete heart block, sudden death, nodal rhythm and chronic atrial fibrillation occurred in seven patients (18%). Two patients have required reoperation for mitral regurgitation. Five have clinically recognizable mild to moderate mitral regurgitation controlled with medical management; twenty-five patients are asymptomatic at current evaluation. Estimated actuarial survival at 13 years is 88 $\pm$ 6%, with an actuarial survival free of reoperation of 82 $\pm$ 6%. However, actuarial survival free of any late complication including late death, reoperation, serious arrhythmia, or mitral regurgitation is only 52 $\pm$ 10% at 13 years.

Proposed Course: These and additional patients as they undergo operation will be included in our continuing evaluation of the natural history of this operatively treated congenital cardiac anomaly.

Presentations:

Goldfaden, Daniel M.: Long-term results of repair of incomplete persistent atrioventricular canal. American Association of Thoracic Surgery. 61st Annual Meeting, May 1981, Washington D.C.

Publications:

Goldfaden, D.M., Jones, M., and Morrow, A.G.: Long-term results of repair of incomplete persistent atrioventricular canal. J. Thorac. Cardiovasc. Surg. (IN PRESS).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02711-02 SU

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Quantification of regional myocardial dysfunction produced by reductions in coronary blood flow: Assessment by sonomicrometry techniques

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

PI: Steven R. Gundry, M.D., Clinical Associate, Clinic of Surgery, NHLBI

Other: Harry W. Seipp, Chief, Technician, Clinic of Surgery, NHLBI
Rodger E. Solomon, Electrical Engineer, Biomedical Engineering, DRS
Michael Jones, M.D., Senior Surgeon & Investigator, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

Biomedical Engineering, DRS

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Clinic of Surgery

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National Heart, Lung & Blood Institute

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Critical coronary artery obstruction reduces coronary blood flow, but the magnitude of coronary blood flow reduction producing myocardial dysfunction is unknown. We examined the quantitative effects of controlled coronary blood flow reductions upon myocardial function using sonomicrometry techniques. The ischemic area of myocardium becomes dilated and hypokinetic when coronary blood flow is reduced more than 25%. Sonomicrometry techniques detect changes in myocardial function with enhanced sensitivity over techniques which measure global myocardial function.

PHS-6040
(Rev. 2-81)

DESCRIPTION: Paired three mm piezo-electric ultrasound crystals were implanted 1.5 cm apart in the left ventricle in the distribution of the left anterior descending coronary artery along the septal-free wall axis of fiber shortening in 7 dogs. An adjustable occluder encircled the left anterior coronary artery above its first diagonal branch; an electromagnetic flow probe, located distal to this occluder measured coronary blood flow. The left anterior descending coronary artery distal to the occluder supplied 36% of the left ventricle. In evaluations on each dog, regional and global left ventricular function was measured before, during, and after reductions in coronary blood flow of 25, 50, 75, 90 and 100%.

RESULTS:

	Control	25%	50%	75%	90%	100%
Diastolic expansion (mm)	1.5±0.5	1.7±0.5	2.0±0.4*	2.0±0.4*	2.2±0.4*	2.1±0.4*
Systolic shortening (mm)	1.4 ±0.4	1.1±0.4	0.8±0.3*	0.4±0.3**	0.4±0.3**	0.3±0.4**
d length/dt (mm/second)	8.6±2.0	7.3±2.0	5.8±3.0*	3.0±2.0*	2.8±4.0*	2.4±3.0*

*$p < 0.04$ **$p < 0.001$

There were no statistically significant changes in LVEDP, dp/dt, aortic systolic pressure, cardiac output, or endocardial ST segments. These results indicate that significant changes occur in regional ventricular wall motion when CBF is reduced more than 25%. The ischemic area of myocardium becomes dilated and hypokinetic. Moreover, these changes occur without significant global left ventricular dysfunction or endocardial ST segment elevations. We conclude that reductions of CBF by more than 25% produces severe regional myocardial dysfunction which was previously undetectable by global ventricular function measurements. Sonomicrometry represents a most sensitive technique to assess regional myocardial function and ischemia.

PROPOSED COURSE: This study has been completed.

PRESENTATION:

Gundry, S. R.: Sonomicrometry to assess regional myocardial dysfunction produced by reductions in coronary blood flow. Surgical Forum, American College of Surgeons, Atlanta, Ga. Octiber 1980.

PUBLICATION:

Gundry, S.R., Seipp, H. W., Solomon, R.E., and Jones, M.: Sonomicrometry to assess regional myocardial dysfunction produced by reductions to coronary blood flow. Surg. Forum XXXI:276-279, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02712-01 SU

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Hemodynamic results following aortic valve replacement with the Hancock standard orifice bioprosthesis

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

PI: A. Michael Borkon, M.D., Clinical Associate, Clinic of Surgery, NHLBI

Other: Charles L. McIntosh, M.D., Ph.D., Senior Surgeon, Clinic of Surgery, NHLBI
Michael Jones, M.D., Senior Surgeon & Investigator, Clinic of Surgery, "
Lewis C. Lipson, Cardiologist, Cardiology Branch, NHLBI
Kenneth M. Kent, M.D., Ph.D., Chief, Cardiac Cath Lab, Cardiology Branch
Andrew G. Morrow, M.D., Chief, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

Cardiology Branch, NHLBI

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National Heart, Lung & Blood Institute

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

In order to improve hemodynamic results, aortic annulus enlargement has been recommended as an adjunct to valve replacement with the Hancock bioprosthesis. In order to determine a need for this additional procedure, postoperative cardiac catheterization data from 73 patients were reviewed. All patients underwent aortic valve replacement with Hancock standard orifice bioprostheses; annulus enlargement was not employed. Residual peak systolic gradient or small effective valve orifice area did not preclude satisfactory clinical improvement. Small diameter Hancock bioprostheses demonstrated acceptable clinical and hemodynamic function when limited to patients with small body surface area. In these instances annulus enlargement is rarely necessary.

PHS-6040
(Rev. 2-81)

DESCRIPTION: Since July 1974 approximately 120 standard orifice Hancock bioprostheses have been implanted in the aortic position. A concerted effort is made by most cardiac surgeons to implant the largest aortic prosthesis to avoid a significant resting or exercise gradient. An aortic annulus enlargement procedure must be performed to allow a larger prosthesis to be inserted in some patients which adds to the complexity and risk of the operation. In order to determine a need for this additional procedure, postoperative cardiac catheterization data from 73 patients were reviewed. All patients underwent aortic valve replacement with Hancock standard orifice bioprostheses; annulus enlargement was not employed. Patients returned to NIH six months postoperatively to undergo examination and cardiac catheterization. In addition to the standard catheterization, isoproterenol infusion was given to 15 patients to evaluate exercise gradients across the prostheses.

RESULTS: Hancock standard orifice bioprostheses have been implanted in the aortic position in 120 patients since July 1974. Postoperative cardiac catheterization was performed in 73 of these patients. Postoperatively, all but 2 patients were NYHA Class I or II. Peak systolic gradient (PSG), cardiac index (CI), body surface area (BSA), and effective valve orifice area (EOA) were determined. Resting data, mean $\pm$ SEM, were:

Size	No	BSA (M^2)	PSG (mm Hg)	EOA (Cm^2)
21	15	1.63 ± 0.03*	10 ± 3+	1.27 ± 0.17#
23	25	1.83 ± 0.04	7 ± 2	1.46 ± 0.11
25	20	1.92 ± 0.04	7 ± 2	1.72 ± 0.20
27	13	1.92 ± 0.05	5 ± 3	1.97 ± 0.06

* = $p < 0.001$ 21 vs 23, 25, 27 + = N.S. # = $p < 0.05$ 21 vs 25,27

CI (2.6 ± 0.1 L/min/M^2) and EOA index (0.86 ± 0.05 Cm^2/M^2) were similar for all sizes.

Isoproterenol infusion (15 pts) increased the peak systolic gradient from a mean of 10 mm Hg to a mean of 44 mm Hg (range 10-85) independent of valve size. Residual PSG or small EOA did not preclude satisfactory clinical improvement. The small PSG found in valve sizes 21 and 23 are satisfactory for patients with small BSA and, therefore, we conclude annulus enlargement is rarely necessary.

Proposed Course: The paper was presented at the American College of Cardiology 30th annual Scientific Sessions in March 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02713-01 SU

PERIOD COVERED

October 1, 1981 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Mitral valve replacement with a Hancock bioprosthesis - A five to ten year follow-up

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

PI: A. Michael Borkon, M.D., Clinical Associate, Clinic of Surgery, HHLBI

Other: Charles L. McIntosh, M.D., Ph.D., Senior Surgeon, Clinic of Surgery, NHLBI
Thomas J. VonRueden, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Andrew G. Morrow, M.D., Chief, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) In order to assess late results with the Hancock bioprosthesis, 111 patients undergoing mitral valve replacement (MVR) either alone (56) or in conjunction with another type of prosthetic valve, prior to 1975 were evaluated. Hospital mortality was 9.9%. Cumulative follow up is 505 patient-years (pt-yrs); mean 5.4 years. Late mortality for isolated MVR is 4.3 ± 1.3% pt-yrs; actuarial survival is 82 ± 6% at five years and 65 ± 11% at ten years. Anticoagulant therapy was employed only for documented emboli or concomitant mechanical aortic prostheses. The incidence of emboli was 3.3 ± 0.9% pt-years for 62 patients with bioprosthses only. Emboli occurred in 22% of patients in atrial fibrillation compared to 3.8% of patients in normal sinus rhythm. Intrinsic mitral bioprosthesis failure occurred in ten patients; two died. Actuarial late survival, free of intrinsic mitral bioprosthetic failure was 99 ± 1% at five years, 92 ± 4% at seven years, 70 ± 12% at nine years, and 61 ± 13% at ten years. Early hemodynamic function continues to be good, although 7 of 18 patients studied late (avg 85 mos) were followed for possible late deterioration. Intrinsic mitral valve destruction secondary to infection has been observed in only one patient. The question remains whether the long-term risk of late intrinsic valve failure and reoperation will outweight the low incidence of emboli and avoidance of anticoagulant related hemorrhage.

PHS-6040
(Rev. 2-81)

DESCRIPTION: The natural history following replacement of either of the atrio-ventricular valves or aortic valves is directly related to the inherent complications associated with the prosthetic valve implanted as well as the severity of disease necessitating cardiac valve replacement. Since July 1970, approximately 600 Hancock bioprostheses have been implanted in all positions at the NIH. This particular prosthesis has been found clinically acceptable because of good hydraulic function in all positions, low indicence of thrombo-embolism without anticoagulants, nonhemolytic design of the valve, resistance to infection and gradual mode of failure. Clinical and late hemodynamic failures of the Hancock bioprosthesis continue to be our major concern regarding this valve now nearing 11 years of clinical use. Late results have been examined in 111 patients who have undergone mitral valve replacement (MVR) prior to 1975. Patients return to NIH six months postoperatively to undergo examination and cardiac catheterization. Thereafter clinic visits are scheduled each year and periodic hospitalization for long-term follow up are recommended. Major complications are recorded and long-term data obtained.

RESULTS: Hancock bioprostheses were implanted in the mitral position in 111 patients prior to 1975. Isolated MVR was performed in 56 patients, and the remaining 55 patients had either an additional bioprosthesis or mechanical prosthesis in another position. The hospital mortality was 9.9%. Cumulative follow up is 505 patient years (pt-yrs); mean 5.4 years. Late mortality for isolated MVR is 4.3 ± 1.3% pt.-yrs; actuarial survival is 82 ± 6% at five years and 65 ± 11% at ten years. Myocardial infarction and congestive failure were the most common causes of death following MVR. Death secondary to intrinsic valve failure was seen in one patient and prosthetic valve infection and dysfunction in one patient.

Anticoagulant therapy was employed only for documented emboli or concomitant mechanical aortic prostheses. The incidence of emboli was 3.3 ± 0.9%/pt-yrs for 62 patients with bioprostheses only. Emboli occurred in 22% of patients in atrial fibrillation compared to 3.8% of patients in normal sinus rhythm. This would suggest that patients in chronic atrial fibrillation should receive anti-coagulant therapy, preferably antiplatelet therapy rather than Warfarin to avoid the reported 5%/yr anticoagulation complication rate.

Cardiac catheterization revealed good early hydraulic function of the Hancock bioprosthesis in the mitral position. As reported last year late hemo-dynamic data indicated progressive stenosis in 7 of 18 patients studied. Three of the seven patients were reoperated upon more than one year ago and the remain-ing four are being followed. The etiology of progressive prosthetic stenosis has been collagen degeneration and calcification in two patients and "stent creep" in one.

Valve failures have occurred in 10 patients (12 valves); one of these patients had a second failure 47 months after reoperation. Two tricuspid valves failed in these patients in addition to the ten mitrals. Nine of these patients were reoperated upon, with two deaths, and the tenth died before reoperation of infection. Actuarial late survival free of intrinsic mitral bioprosthetic failure was 99 ± 1% at five years, 92 ± 4% at seven years, 70 ± 12% at nine years, and 61 ± 13% at ten years. Modes of failure were gradual and were secondary to collagen degneration, calcification, thrombosis and "stent creep."

Seven patients in our series have had documented sepsis with no prosthetic involvement requiring removal of the prosthesis or recurrence of infection once antibiotics sere discontinued. One patient developed three episodes of endocarditis after triple valve replacement with Hancock bioprostheses; all three valves were destroyed when examined at necropsy 40 months after implantation. No postoperative Mycobacterium chelonei infections have occurred in our series. Hemolysis has not been seen following atrioventricular replacements and perivalvular leaks are uncommon with Hancock bioprosthesis.

It is unknown at the present time whether the long-term risk of late intrinsic valve failure and reoperation will outweigh the low incidence of emboli and avoidance of anticoagulant-related hemorrhage. Until further information becomes available, the Hancock bioprosthesis is used for MVR only in patients older than 60 years or in patients with contraindications for anticoagulant therapy.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02714-01 SU

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Bioprosthetic valvular failure. Clinical and pathological characteristics in an experimental animal model.

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

PI: Glenn R. Barnhart, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Michael Jones, M.D., Senior Surgeon, Clinic of Surgery, NHLBI

Other: Altagracia M. Chavez, M.D., Clinical Associate, Clinic of Surgery,NHLBI
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Tokuhiro Ishihara, M.D., Ph.D., Guest Worker, Pathology Branch, NHLBI
Victor J. Ferrans, M.D., Ph.D., Chief, Ultrastructural Section,
Jack Butane, M.D., Pathology Branch, NHLBI
William C. Roberts, M.D., Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

Pathology Branch, NHLBI

LAB/BRANCH
Clinic of Surgery

SECTION

INSTITUTE AND LOCATION
National Heart, Lung & Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	2	

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) The purpose of this project is to develop an animal model to assess the pathologic alterations and the hemodynamic dysfunction resulting from those alterations of cardiac bioprosthetic valves. Bioprosthetic valves implanted in juvenile sheep demonstrate the same pathologic alterations of degeneration and calcification as those implanted in humans, but these alterations occur in a much shorter period of time in valves implanted in sheep. Bioprosthetic valves of clinical quality from all commercial sources in this country have been implanted in the tricuspid and mitral positions of sheep to evaluate the pathogenesis of bioprosthetic valvular degeneration and to compare the characteristics and severity of these alterations in the different types of bioprosthetic valves.

PHS-6040
(Rev. 2-81)

DESCRIPTION: The development of an ideal cardiac valve substitute remains a major problem in cardaic surgery. Bioprosthetic cardiac valves have become the valves of choice at many institutions, primarily because they do not require chronic anticoagulant therapy. However, it has become apparent that the long-term durability of bioprosthetic valves is finite; degeneration and calcification of these valves have become major complications of long-term implantation.

We compared the clinical, hemodynamic and morphologic findings in 18 young sheep in which procine valvular bioprostheses (8 animals) and bovine pericardial bioprostheses (10 animals) were implanted in the tricuspid position. At the time of terminal elective studies (mean of 5.2 ± 0.2 months after implantation), 6 animals had ascites, 16 had hepatic congestion and 4 had bioprosthetic valvular infection. Hemodynamic studies (n = 10) showed that the tricuspid transvalvular mean diastolic gradients were not different at implantation and at termination of the study (4.7 ± 0.8 vs. 4.9 ± 0.9 mm Hg); however, tricuspid valve end-diastolic gradients increased from 1.2 ± 0.6 mm Hg to 3.9 ± 0.5 mm Hg ($p < 0.01$). Each of the 18 valves had calcific deposits. Quantitative studies revealed that implanted porcine valvular bioprostheses (n = 7) contained a mean of 323 ± 165 mg of calcium/gm of dry weight of cuspal tissue, in contrast to 0.2 mg/gm in unimplanted porcine valvular bioprostheses. Similarly, implanted bovine pericardial bioprostheses (n=6) contained a mean of 421 ± 115 mg of calcium/mg of dry weight of cuspal tissue, in contrast to 0.3 mg/gm in unimplanted bovine pericardial bioprostheses. Morphologic findings in both types of bioprostheses included calcific deposits, collagen degeneration, leaflet immobilization and retraction, and fibrous sheaths. The latter were more extensive in bovine pericardial bioprostheses than in porcine valvular bioprostheses. We conclude: 1) that the pathological alterations which develop in bovine pericardial bioprostheses are generally similar to those in porcine valvular bioprostheses, but may be more severe; 2) that these alterations lead to physiological and clinical sequelae similar to those of bioprosthetic valvular failure in humans, and 3) that young sheep constitute an excellent experimental model for *in vivo* testing of bioprosthetic cardiac valves.

Proposed Course:

Papers submitted for publication:

Barnhart, G.R., Jones, M., Ishihara, T., Chavez, A. M., Rose, D. M., and Ferrans, V.J.: Bioprosthetic valvular failure. Clinical and pathological characteristics in an experimental animal model. J. Thoracic Cardiovasc Surg.

Barnhart, G. R., Jones, M., Sihihara, T., Rose, D.M., Chavez, A. M., and Ferrans, V.J.: Degeneration and calcification of bioprosthetic cardiac valves. Am. J. Pathol.

Further studies of this project include a comparison of the pathologic alterations that occur in the mitral and tricuspid position, a comparison of the pathologic alterations that occur in bioprosthetic tissues implanted in the subcutaneous and intracardiac position, and a study of the morphology of the infection of bioprosthetic valves.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02715-01 SU

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Microembolization and left ventricular function during cardiopulmonary bypass

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

PI: Karl J. Karlson, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Michael Jones, M. D., Senior Surgeon, Clinic of Surgery, NHLBI

Other: Robert Rutledge, M. D., Clinical Associate, Clinic of Surgery, NHLBI
James N. Ganey, M.D., Guest Worker, Clinic of Surgery, NHLBI
Andrew G. Morrow, M.D., Chief, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)
none

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INSTITUTE AND LOCATION
National Heart, Lung & Blood Institute

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) The production of microemboli and altered left ventricular (LV) function was studied in 24 adult sheep during 4 hours of cardiopulmonary bypass (CPB), using membrane (12 sheep) and bubble oxygenators (12 sheep). Arterial pressure, HR, CVP, and dP/dt were comparable in the two groups. Active compliance curves of LV intracavitary balloon volume vs. filling pressure were determined every 30 minutes. Ultrasonic on-line counts of particulate and bubble microemboli were made at 3 sites: venous line, distal to oxygenator, and arterial line. At the onset of CPB, bubble oxygenators produced 62,000 ± 14,000 microemboli/liter of flow (74% bubbles, 26% particles) and membrane oxygenators produced 1360 ± 580 microemboli/liter of flow (88% bubbles, 12% particles). Over 4 hours of CPB, embolization 71% in the bubble oxygenator group secondary to particles; whereas, embolization 61% in the membrane group. LV dV/dP was 1.6 ± 0.2 cc/mmHg at the onset of CPB, and 42% in the bubble group and 23% in the membrane group over 4 hours (p .05). We conclude that bubble oxygenators produce more microemboli than membrane oxygenators do, and that these microemboli may contribute to a decrease in LV compliance during prolonged CPB.

PHS-6040
(Rev. 2-81)

DESCRIPTION: This project has demonstrated that microembolization occurs from oxygenators in the circuits of heart-lung machines. There is a difference in both the quality and quantity of microemboli between bubble oxygenators and membrane oxygenators. Bubble oxygenators produce almost fifty times more microemboli than membrane oxygenators do. We have shown that there is a correlation between the amount of embolization and the subsequent decrease in left ventricular function which occurs after prolonged cardiopulmonary bypass. Therefore, in order to decrease the deleterious effects of prolonged cardiopulmonary bypass during complicated open-heart operations, it would be prudent to select oxygenators which would produce the fewest microemboli possible (i.e. membrane oxygenators).

Proposed Course: The results of this experiment have been submitted to the American Heart Association for presentation at the annual meeting in November 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02716-01 SU

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
LATE RESULTS AFTER OPERATIONS FOR LEFT VENTRICULAR OUTFLOW TRACT OBSTRUCTION

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

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Thomas J. Von Rueden, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Andrew G. Morrow, M. D., Chief, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

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National Heart, Lung & Blood Institute

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

We have operated upon more than 130 children and young adults for congenital forms of left ventricular outflow tract obstruction. The present study involves the late results of these operations performed at our institution and a review of the published results from other institutions. Patients included in the study were aged 1-18 years old at operation who had been followed five or more years postoperatively. Forms of left ventricular outflow tract obstruction included valvular aortic stenosis, discrete and diffuse fibrous subaortic stenosis, and muscular subaortic stenosis. Operative mortalities were low (0-6%). However, the operations appear to be palliative ones because only approximately 50% of patients have satisfactory late results.

PHS-6040
(Rev. 2-81)

DESCRIPTION: We evaluated the late results of operations for the relief of left ventricular outflow tract obstruction (LVOTO) in young patients, 1-18 years old, from our institution who have been followed for at least five years and from studies in the recent literature which had average follow up durations of five or more years. Operative mortalities for our series and those series we reviewed were low: 1.9% of 522 patients with valvular aortic stenosis (VAS), 6.0% of 222 patients with fixed subvalvular aortic stenosis (SAS) and 5.5% of 18 patients with hypertrophic subaortic stenosis (HSS). From our series, gradients early postoperatively were decreased to less than 50 mm Hg in 88% (30/34) with VAS, in 68% (15/22) with SAS and in 88% (8/9) with HSS. Late survivals for patients in the combined series were: 90% (472/522) for VAS, 86% (190/222) for SAS and 82% (14/17) for HSS, after mean follow up periods of 5-14.4 years. All of our late survivors have had symptomatic improvement; 95% (58/61) are asymptomatic. However, for our patients actuarial analysis predicts that 50 $\pm$ 8% of those with VAS and 44 $\pm$ 10% of those with SAS after ten years will be free from the adverse postoperative events of residual and/or recurrent LVOTO, clinically significant aortic regurgitation, reoperation, endocarditis or late death. Using the same adverse postoperative events to determine satisfactory late results from the combined series, we found that 54% (281/522) of those operated upon for VAS, 54% (120/222) of those operated upon for SAS and 78% (14/18) of those operated upon for HSS had satisfactory late results 5 - 14 years after operation. Of our patients having unsatisfactory late results, major hemodynamic abnormalities were detected in 55% (23/42) within one year postopertively. Thus, it appears that operations for most children with LVOTO are palliative ones. These patients should have early postoperative assessments and continuing long-term follow up evaluations during childhood, adolescence and adulthood.

PROPOSED COURSE: The patients in this study will continue to be followed and additional patients will be added to the study as they undergo operations. The purpose of the on-going studies is to further delineate the natural history of these operatively treated cardiac abnormalities with the objectives of eliminating or alleviating unsatisfactory late results.

Publications:

Jones, M., Barnhart, G. R., and Morrow, A. G.: Late results after operations for left ventricular outflow tract obstruction. Am. J. Cardiol. (submitted)

Presentations:

Von Rueden, T. J., Jones, M., and Morrow, A.G.: Operations for congenital valvular aortic stenosis: Observations 5-21 years later. Presented at 53rd Scientific Sessions of American Heart Association, November 1980. Abstract published.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02717-01 SU

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Influence of Minoxidil on regional myocardial blood flow and cardiac pathology in beagle dogs

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

PI: G. Kimble Jett, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Michael Jones, M.D., Senior Surgeon, Clinic of Surgery, NHLBI

Other: Virginia Kuentz, Technician, Clinic of Surgery, NHLBI
Victor J. Ferrans, M.D., Chf, Ultrastructural Sec., Pathology Br,NHLBI
G. Herman, Ph.D., Food and Drug Administration, PHS

COOPERATING UNITS (if any)
Ultrastructural Section, Pathology Branch, NHLBI
Food and Drug Administration, PHS

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) Minoxidil is a potent, long-acting peripheral vasodilator used for treatment in patients with severe hypertension. Although minoxidil has a low order of toxicity, it has been found to cause several types of pathologic alterations in the heart consisting of left ventricular and right atrial fibrosis. The etiology of these pathologic alterations is believed to be due to hemodynamic changes caused by minoxidil.

We measured the influence of minoxidil on regional myocardial blood flow and attempted to correlate blood changes with histologic changes in these same regions. We found that minoxidil significantly increased myocardial blood flow to all regions of the heart except to the anterior and posterior papillary muscles of the left ventricle. Minoxidil significantly decreased mean aortic pressure, but significantly increased heart rate and cardiac output. Interestingly, LVEDP, pulmonary artery wedge and right sided pressures were significantly increased with minoxidil treatment. Although the histologic examinations are still being performed, it can be speculated that the previously seen myocardial necrosis of the papillary muscles may be on an ischemic basis with an increased work and end-diastolic pressure of the left ventricular but constant myocardial blood flow.

PHS-6040
(Rev. 2-81)

DESCRIPTION: Adult beagle dogs weighing between 8-12 Kg were utilized. Ten dogs were given two doses of minoxidil (3 mg/Kg) 12-24 hours apart. Shortly thereafter the dogs were anesthetized with alpha chlorolose and the heart exposed through a left thoracotomy. Aortic, left atrial and left ventricular pressures were recorded. Pulmonary artery, right ventricular and right atrial pressures as well as cardiac output were measured utilizing a Swan-Ganz catheter inserted through an internal jugular vein. Cardiac output was calculated using the thermal dilution technique. Regional myocardial blood flow was measured using a radioactive microsphere (^{46}Sc) 9 microns in diameter. At the conclusion of the study, the heart was excised and the fat and coronary vessels were discarded. The atria were divided into right and left atrial sinus and appendage regions as well as interatrial septum. The ventricles were divided into right, left, right sided septum and left sided septum. The right ventricle was subdivided into outflow and body regions. The left ventricle was subdivided into anterior and posterior walls. The anterior and posterior papillary muscles were also isolated. In addition to myocardial blood flow, each region of tissue underwent complete histologic examination.

A second group of 10 dogs were given a placebo instead of minoxidil. These animals served as control and were used for comparison with the minoxidil treated group.

Results:

Blood (cc/min/gm)

	RA	
	Sinus	Appendage
Control	*1.54 ± .28	1.39 ± .14
Minoxidil	**2.74 ± .22	**3.12 ± .34

			RV	
	Ant. Pap.	Post. Pap.	Outflow	Body
Control	1.90 ± .21	1.85 ± .10	1.22 ± .10	1.30 ± .20
Minoxidil	2.32 ± .41	2.21 ± .25	**3.41 ± .19	**3.59 ± .22

	LV	
	Anterior	Posterior
Control	1.38 ± .05	1.48 ± .07
Minoxidil	**2.53 ± .11	**2.96 ± .11

Hemodynamics

	HR	CO	Ao	$\overline{Ao}$
Control	134 ± 5	2.6 ± .2	125/88 ± 4/3	104 ± 4
Minoxidil	**152 ± 4	**4.5 ± .2	**118/60 ± 7/2	** 79 ± 6

	LVEDP	PAW	RV	RA
Control	5 ± 1	5 ± 1	20/2 ± 2/1	2 ± 1
Minoxidil	**14 ± 1	**13 ± 1	**30/7 ± 2/1	**8 ± 1

* Mean ± Standard Error of the Mean ** p 0.05

Proposed Course: Once the histologic evaluation is completed the results of this study will be submitted for publication.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02718-01 SU

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Mitral valve replacement after closed mitral commissurotomy

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

PI: Robert Rutledge, M.D., Clinical Associate, Clinic of Surgery, NHLBI

Other: Leland Siwek, M.D., Clinical Associate, Clinic of Surgery, NHLBI
J. B. Zwischenberger, M.D., Clinical Associate, Clinic of Surgery, NHLBI
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Karl J. Karlson, M.D., Clinical Associate, Clinic of Surgery, NHLBI
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COOPERATING UNITS (if any)

LAB/BRANCH Clinic of Surgery

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INSTITUTE AND LOCATION
National Heart, Lung & Blood Institute

TOTAL MANYEARS: 3/4	PROFESSIONAL: 3/4	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)

Closed mitral commissurotomy (CMC) was performed on 232 patients (67M, 165F) with isolated mitral valve disease from 1954-1980. Mean age was 41 yrs. and operative mortality was 4%. Pre- and postop catheterization was obtained in 70% of patients. Mean NYHA Functional Class preop was 2.7 and improved to 1.6 after CMC. Mean mitral valve gradient (MG) decreased from 15 $\pm$ 0.5 to 6 $\pm$ 0.4 ($p < .001$). FC was 1.6 and 1.8 at 5 and 10 yrs after CMC; 20 patients (10%) underwent more than one CMC. Actuarial survival free of mitral valve replacement (MVR) was 83 $\pm$ 3% and 66 $\pm$ 4% at 5 and 10 years. MVR was required in 42 patients (15M, 27F) a mean of 9 years after CMC (range 1-26 yrs). In this subgroup the CMC initially improved FC from 2.7 to 1.9 and MG from 13 $\pm$ 0.8 to 6 $\pm$ 0.9 ($p < .001$). Patients subsequently undergoing MVR had a higher mean LA pressure after CMC than those who did not require MVR (18 $\pm$ 1.2 vs 13.5 $\pm$ 0.6, $p < .001$). Operative mortality for MVR was 12%. MVR improved FC from 3.1 to 1.7 and MG from 12 $\pm$ 1.1 to 5 $\pm$ 0.6 ($p < .001$). At both 5 and 10 years after MVR FC was 2.0. CMC provides useful palliation wven in 20% of patients who ultimately require MVR.

PHS-6040
(Rev. 2-81)

DESCRIPTION: A recent lead article in the Annals of Thoracic Surgery reviewed the experience after closed mitral commissurotomy in 139 patients. Much of their report generated concern because of some difficulties with their data, i.e. they reported pre- and postop follow up but no other follow up data; they reported 3 late deaths - supposedly with 25 year experience; no cath data was presented except for preoperative LVEDP; they reported 18 patients who underwent mitral valve replacement after CMC with a 20% mortality.

We felt that the experience at the NIH provided a much greater amount of useful information on patients undergoing CMC and also of the subgroup coming to MVR. Although three previous papers from the NIH have examined the results of various subgroups or specific hemodynamic effects of CMC, these appeared in 1962, 1969 and 1972. We felt that with greater long-term follow up and the use of sophisticated statistical analysis would provide a worthwhile addition to the present knowledge about CMC.

Proposed course: Abstract has been submitted to American Heart Association for November 1981 Scientific Sessions.

ANNUAL REPORT OF THE
LABORATORY OF TECHNICAL DEVELOPMENT
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
OCTOBER 1, 1980 TO SEPTEMBER 30, 1981

In a program applying a basic science concept of instrumentation this laboratory recognizes advances in basic physical science and then evaluates their potential to serve biomedical science. Instrumentation for measurement, separation or control of biosystems of interest to biomedical research form the basis for interlaboratory cooperation. Instrumentation concepts are selected for their promise of contribution to advancement of bioscience in general or in response to anticipated requirements.

Significant progress has been made this year in the development of new microchemical analysis instruments. This progress has been connected with developments in colorimeters and fluorometers with working volumes of 300 nL or less and the exploration of an unusual luminescence technique for the measurement of ammonia at picomole levels. Part of the basis of this progress is a new one-piece colorimeter cuvette for optical absorbance measurements. The working volume of the cuvette is 220 nL and it has a path-length of 1 cm. The colorimeter using this cuvette is part of a continuous flow analysis system. A stream of reagent flows past a port where samples are injected. A color producing reaction occurs that is specific for the substance to be analyzed. The colored product passes through the cuvette where the optical transmittance, proportional to the amount of substance present, is measured. The performance of the instrument has been demonstrated on four materials of interest to renal physiologists: calcium, magnesium, phosphate, and urea. One can easily achieve picomole sensitvity for these substances. Careful attention to details such as sample injection procedure and fluid path cleanliness permits even greater sensitivity. For example, measurement of total calcium in 5 nL samples of tubule perfusion fluid can be measured with a precision of 1.1% at a total content of 5 picomole. In addition to the facility with which high sensitivity chemical analyses can be performed the samples can be recovered for isotopic analysis, and in this way, multiple analysis can be made on the same sample. The new microcolorimeter is very simple and compact compared with present microanalytic instruments and it is compatible with a wide range of chemical analyses.

Fluorometric techniques are sometimes more sensitive or specific than colorimetric methods. A new microfluorometer is under development. This instrument, which has a cuvette with a volume of 300 nL, uses a fiber optic to carry excitation light from the light source to the sample. A highly efficient collection scheme directs the light to the photomultiplier detector. The fluorometer has demonstrated the ability to detect fluorescein at a concentration of 10^{-9} M/L and NADH at 10^{-7} M/L. When the fluorometer is part of a continuous flow analysis scheme, it can detect approximately 10^{-15} moles of fluorescein injected into the stream. By adjusting the pumping rate, the continous flow system can be

used for enzymatic analyses. The delay between injection and detection permits the enzymatic analyses. The delay between injection and detection permits the enzyme reaction to procede so that concentration changes in a fluorescent material, such as the cofactor NADH, can be used to quantitate the substance being analyzed. Thus, the microfluorometer complements the microcolorimeter.

A more unconventional technique is being explored as an analytical system for picomole amounts of ammonia. Mercury vapor in nitrogen can be excited by irradiation with light from a mercury lamp. The excited mercury atoms combine with ammonia present in the gas mixture to form an excited complex which dissociates with the release of light. The amount of light at the appropriate wavelength is proportional to the amount of ammonia. Ammonium in fluid samples can be converted to ammonia gas by putting the samples in contact with the appropriate release agent. A stream of mercury vapor in nitrogen sweeps the released ammonia to the detector. The technique has demonstrated the ability to detect less than 3 picomoles of ammonia. Further work is being carried on to reduce interference from oxygen and water vapor and to increase the sample throughout.

These new microchemical analysis methods make it possible to perform easily many sorts of biochemical studies on small bits of tissue or cloned cells in tissue culture. With these tools, understanding of the physiology and biochemistry of the subunits of complex organs such as the kidney can be advanced more rapidly.

Work on methods and apparatus for the study of kidney and toad bladder epithelial cells grown as sheets on porous membranes continues to yield important results. The central element is the porous bottomed culture device (PBCD) in which the membrane is cemented to polycarbonate rings which provides electrical and chemical isolation between the solutions contacting each side of the membrane. With these PBCD's new knowledge of development of epithelia is being obtained.

Spacing the PBCD's off the bottom allows exchange of nutrients and waste from both sides and results in a response to vasopressin by A6 cells (from Xenopus laevis kidney) which does not occur otherwise. However, the cells continue to grow to the limits of this nutrient supply and eventually become very sensitive to stirring and medium replacement. This growth can be controlled by using medium with no serum added on the apical side (urine side) and the usual medium 10% fetal bovine serum on the basal side (blood side). This elimination of the serum on the apical side not only controls growth but also speeds up the development of the potential differences (PD) across the epithelia and increases the PD. The ability to manipulate the 2 sides of the preparations separately offers great promise for the study of the development of epithelia. This is particularly true when one uses transparent support membranes such as the collagen membranes which we have developed. One can watch the A6 cells spread across the membrane and become confluent. The PD does not develop at this time. The cells go

through many gyrations (moving and changing shape) before the PD develops a couple of days later. These gyrations are much simplified and shortened when medium with no serum is used on the apical side.

The growth of medullary thick ascending limb MTAL cells (from rabbit kidney) on our collagen membrane PBCD's continues. These cells on collagen have shown many of the characteristics of the MTAL in the rabbit: sensitive to Furosimide, insensitive to Amiloride, insensitive to vasopressin (at mamaliam concentrations) and a PD which is positive on the apical (lumen) side.

Due to the important role which calcium ions appear to play in many cells including epithelial cells, a project has been started with the objective of improving the specificity of the $Ca^{\#}$ ion selective electrode. The calcium activity inside most cells is very small: less than 10^{-6} molal. In fact, most experimental values are probably too high. Values obtained using dyes being high because these dyes have affinities for $CA^{\#}$ comparable to some of the things inside the cells which bind the $CA^{\#}$. The ion specific electrode reads high due to interferences of other ions such as K^{+}, Na^{+}, and $Mg^{\#}$. These inferences are particularly bad for small microelectrodes needed to successfully enter epithelial cells (<0.1 micron tip diameter). The changes being explored to obtain improved specificity are: (1) Uses of glases without diffusable ion (fused quartz and Corning 1723). (2) Silanization of the glass with monochloro-silanes with long hydrocarbon chains with careful exclusion of water vapor. (3) Explore attachment of the $Ca^{\#}$ exchange molecules directly to the glass.

Fluorescence spectroscopy is a dynamic technique involving the measurement of multiple parameters such as lifetime, spectra, polarization and quantum yields. We have extended the usefulness of fluorescence spectroscopy by developing new methods of assay and by exploring the limits to which established methods can be applied.

We have developed a fluorometric assay for phospholipases, enzymes that cleave phosphatidylcholine (lecithin). The method uses phosphatidylcholine liposomes containing concentrated 6-carboxyfluorescein. The dye-liposomes are prepared by sonication followed by Sephadex filtration to remove unincorporated dye. The liposomes are nearly non-fluorescent until they are lysed; then, the dye is released and concentration quenching is removed. When these liposomes are exposed to phospholipases in the presence of Ca^{++}, the amount of fluorescence is related to the amount of enzyme. Conventional assays of phospholipases are cumbersome (titrimetry, radioisotopic assay of chromatographed products), but the fluorometric assay is rapid, sensitive, and can be used in rapid kinetic studies. Lifetime studies and absorption spectra show that the concentration quenching may be due to dimerization of the dye and energy transfer to non-fluorescent dimers.

It was found that bovine serum albumin (BSA) activated and inhibited

phospholipases from different sources. Both BSA and fatty acids activated bee venom phospholipase A_2. Pancreatic phospholipase A_2 and phospholipases C from bacteria were inhibited by BSA, while snake venom enzymes were activated. The action of serum albumin on these enzymes probably has physiological significance, and the activation and inhibition may represent evolutionary adaptations favoring the predator in one instance, and favoring host defense against autolysis or bacterial invasion in the other.

The liposome-dye system also is the basis of a very sensitive assay for membrane-active peptides from insect venoms. Exposure of liposomes to bee venom mellitin can be detected. The assay is rapid compared to the quasi-bioassays now used, which require the assessment of mast cell degranulation by these membrane-active peptides. The new assay has been applied to effluents from HPLC runs on wasp venom.

Stopped-flow fluorescence has been useful for defining the rates of labeling of proteins by dyes. Very little is known of these rates, since there is usually no easy way to follow labeling without removing free and bound dye. We approached the problem by using the o-phthalaldehyde system which becomes fluorescent only when the dye attaches to proteins or primary amines. The rates we find are in the milliscond to second range and are faster than one would predict on diffusional considerations. Apparently, binding of the dye by protein facilitates the rate of labeling.

The rate of acetylation of proteins can be followed. BSA has been found to be acetylated by 4-methylumbelliferone acetate, with the resultant liberation of the fluorescent 4-methylumbelliferone. The rate of acetylation is much slower than the rate of reaction with o-phthalaldehyde, and this no doubt reflects the different sites and types of reaction involved. This phenomenon can serve as a model of physiological acetylations, such as the acetylation of albumin and other proteins by aspirin.

We demonstrated the versatility of countercurrent chromatography in separation and purification of biological materials. The method allows cleaning-up and enrichment of small amounts of biological materials present in a large volume of biological fluids or crude extracts in a short period of time without significant loss of samples. The method also provides efficient separation of samples in both analytical and preparative scales. A countercurrent chromatograph for large scale preparative separations was developed and successfully tested on separations of one gram quantities of dinitrophenyl amino acids. The scheme can be easily scaled up by the use of larger and/or longer columns. Separations of samples ranging from microgram to several hundred milligram quantities can be efficiently performed with several schemes of countercurrent chromatography each having its own specific advantage. The new horizontal flow-through coil planet centrifuge has a pair of separation columns, one suitable for preparative-scale separations and the other, capable of efficient

analytical-scale separations. The toroidal coil planet centrifuge is a compact table top model of a countercurrent chromatograph which enables efficient analytical-scale separations with a variety of two-phase solvent systems. The non-synchronous flow-through coil planet centrifuge is ideal for separation of cells and macromolecules with Albertsson's polymer phase systems. Each scheme has been applied to separations of biologically important samples to prove the usefulness of countercurrent chromatography.

The Section on Pulmonary and Cardiac Assist Devices is concerned with devising methods, devices, and techniques to assist the failing lungs and the failing heart. The cardinal approach lies in resting the diseased organs to "buy time" for the diseased lungs and the diseased heart to heal under more optimum conditions than otherwise possible, and averting circumstances under which healing may no longer be possible. To provide an environment for the healing of either organ this environment should not contribute to the progression of the disease. For instance, changing ventilator settings on a patient with Adult Respiratory Distress Syndrome (ARDS) may result in a transient, improvement in blood gases, but ultimately can contribute to the worsening of the disease, and death. Hence, the use of the aritifical lung and the booster heart of what ever type must assist the failing organs and not do harm.

Earlier work in this Section had led us to explore benign methods of managing acutely diseased lungs. These studies evolved from our capability of removing through an artificial lung, connected to a veno-venous circuit, a major fraction of total metabolically produced carbon dioxide. This method allowed us to ventilate the diseased lungs at low pressures, low tidal volumes, and at low rates, as the need for keeping $PaCO_2$ within normal range was simplified by the extracorporeal removal of CO_2 by the artificial lung. Our laboratory was first to show that such lungs recover in short order when treated as described.

In cooperation with the University of Milan, this concept of lung management was recently applied to 23 consecutive patients with ARDS, all meeting ECMO (Extracorporeal Membrane Oxygenation) criteria (10% survival). Eleven of those patients were refractory to all forms of lung management and further aggressive state of the art ventilator treatment was judged not likely to produce benefit. There was immediate improvement in the patients' pulmonary status upon placement on $ECCO_2R$ with LFPPV (Low Frequency Positive Pressure Ventilation). Within the first hour of such treatment, most of the patients were sufficiently improved to have met ECMO criteria for terminating bypass, although bypass for the improvement of lung mechanics had to be continued for a mean of 6 1/2 days. The overall survival of this series was 82%.

We are continuing to explore means for managing preterm lungs of newborns at high risk of developing RDS (Respiratory Distress Syndrome). Studies using fetal lambs of 128-130 days gestation have shown that initial insufflation to a pressure of 35 cm H_2O pressure and a prolonged hold

for 5 seconds results in a substantial reduction of perinatal pulmonary mortality. Addition of a constant intrapulmonary distending pressure of 15 cm H_2O results in a gradual rise in lung compliance (TLC). At a point where TLC exceeds 0.6 ml $(Cm\ H_2I)^{-1}\ min^{-1}$ the fetal lambs can be safely delivered, the umbilical cord tied, knowing that RDS will not evolve. The L/S (Lethecin/Sphingomyelin) ratio of amniotic fluid in this age group is a mean of approximately 0.6, well in the highly immature range. And yet, we have shown that none of our animals that passed this treatment protocol went on to develop RDS. The L/S ratio of saline lung wash of fetal lambs so treated, and following 24 hours of mechanical ventilation, showed an L/S ratio well in excess of 2.5 i.e. in the fully mature range. The minimum surface tension of lung fluid, and saline lung wash was and remained in the abnormal range for new borns, yet was clearly sufficient to allow mechanical ventilation to proceed normally once pulmonary conditioning was complete. Recently studies have employed the use of an extracorporeal artificial lung to facilitate the conditioning of yet younger fetal lambs. Preliminary results are encouraging.

Our fetal lamb laboratory studies lead us to believe that RDS of the newborn is a preventable, treatable, and curable disease. Bronchopulmonary dysplasia resulting from protracted mechanical ventilation can be aborted through the application of techniques learned from these studies.

The section has further explored the use of a continuous cell separator of our design that eliminates the use of rotating seals. Malaria parasites were separated from rhesus monkey blood, with a 20 fold enrichment in parasite concentration in the harvested sample. This blood centrifuge is particularly desirable for this purpose, as its total priming volume, including rotor and blood circuit, is but 110 ml, all of which can be returned to the animal. The blood centrifuge has also been used in pilot studies for the separation of sickle RBC from normal donor RBC. The great versatility of the rotor head design allows for either single or double stage separation. The latter being especially useful for on-line high purity platelet concentration, without contamination by lymphocytes.

In the Section on Biophysical Instrumentation, work is continuing on the development of instrumentation for one of the major unsolved problems of biological interest involving hemeproteins which is, the mechanism of the ligand or electron transport reaction at the active site of the protein. The question is whether this is purely a chemical reaction and if so, what is its nature or is it, in the case of hemoglobin and myoglobin, simply, an entrapment and isomerzation much as in the cryptands? To assist the molecular biologist in answering these questions, Dr. Balko has been developing a low temperature Mossbauer classical transmission instrument. In the classical method the probe of the energy levels in the nucleus by the gamma ray is done by adding or subtracting a small amount of energy to the probing gamma rays by moving the source, i.e. doppler shift in the energy. While this is a necessary first approach, it does not delineate the underlying mechanisms of energy exchange in the nucleus which are so

profoundly affected by the environment of the outer shell electrons. Using SEDM and directly probing a particular Mossbauer line and observing the energy exchange to the other Mossbauer line in the nucleus this mechanism can be elucidated. This means that, in the case of hemoglobin, each intermediate can be observed and the contribution of each to the Gibbs Free energy which involves the iron-porphyrin can be ascertained. Both of these instruments have now been completed to the point that we have demonstrated their usefulness in several cases of biological interest.

The heme involvement in cooperativity of hemoglobin was investigated using transmission Mossbauer spectroscopy. Strained and unstrained forms of model compounds of myoglobin and normal and modified hemoglobins enriched in ^{57}Fe were studied, low temperatures (4.5k) and strong magnetic fields (50K Gauss) were used to split the spectra, and line shape theory utilizing superoperator technique was used to analyze the data. At least two different heme sites for iron were found in HbA and a new heme structure similar to that of an unstrained model compound was found in the modified Hb system with characteristics similar to Hb. Osler. Bleomycin, a glycopolypeptide with cytotoxic properties, was investigated and two nonequivalent iron sites were found. Electronic relaxation in the Mossbauer spectra differentiated the active from the inactive.

A new picosecond spectrometer has been built in an attempt to study the detailed movement of the energy and structure of the protein in the reaction of hemoglobin with carbon monoxide or oxygen. The design and construction has been completed. Experiments are underway for applying this technique to a series of biochemical problems. A serious impairment to the application of a vidicon to detect transient events has been observed and well characterized. A technique has been devised which overcomes this impairment as well as significantly advancing the area of vidicon imaging to pulsed specroscopy.

The binding of Ca(+2) and Li(+) to the inophore 211 has been made. The reaction was found to proceed via the mechanism $M^{n+} + L = (ML)_1{}^{n+} = (ML)_2{}^{n+}$. Both kinetic and thermodynamic data have been obtained for this reaction using the one instrument. Additionally this binding mechanism is representative of many enzyme-substrate reactions and suggests the wide applicability of the TSF technique to biochemistry. The binding of Ca(+2) to the widely used buffer EGTA has been investigated. The rate constant for complexation has been observed to be $10^6 M^{-1} S^{-1}$ at physiological pH. This rate is quite slow and unexpected proving quite important to the accurate use of EGTA as a calcium buffer. In order to further reduce artifacts in the thermal stopped flow apparatus an improved ball mixer has been developed which reduces the pressure drop by 30% and the flow heating by a factor of ten due not only to the mixer but also to a reduction in right angle turns and an enlargement of entry ports. Trials have begun on a low temperature quenching system using this mixer and a single commercially available stepping motor dilutor. A resolution of 5 milliseconds has so far been obtained.

In the measurement of blood flow our doppler scatter laser light instrument has proved to be useful for studies of muscle blood flow in a cooperative application study with the Neurology Institute and improvements in the instrument developed in the past year are expected to be included in the commercial clinical study instrument that will be available in the near future. With the availability of the commercial version of this instrument our work will be discontinued.

In the measurement of cerebral blood flow by nuclear magnetic resonance this contract program with the Medical College of Wisconsin has terminated in favor of grant support. The facility at the Medical College of Wisconsin now has the facilities and personnel to carry on this project without further contribution from this laboratory. Some continued informal collaboration in the development of electronic circuits will continue.

An extension radio-angio-graphic catheter that will enter small tortuous vessels for embolization or other interventional therapy has been developed to the point of meeting the specifications and is ready for animal testing. A concept for a prosthetic urinary sphincter has been constructed according to the concept and meets preliminary criteria for testing in animals.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01404-13 LTD

PERIOD COVERED October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Membrane Lung Systems for Long Term Respiratory Support

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

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TOTAL MANYEARS:	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 section has further explored some newer approaches to the management of acute lung failure in newborns and in adults. We have postualted that mechanical ventilation by and itself contributes to the evolution of ARDS. We have proposed a treatment approach whereby the pulmonary ventilation of patients with ARDS is reduced to but 2-4 breaths/min, with CO_2 removed by an extracorporeal membrane lung. This results in rapid reduction in VA/Q mismatch, in prompt improvement in PaO_2 and recovery from ARDS.

Work in animal model has shown that lungs of preterm fetal lambs expand rapidly after intrapulmonary insufflation of the lungs, followed by apneic oxygenation. Extracorporeal removal of CO_2 with an efficient membrane lung is a powerful method to control ventilation in these premature newborns.

PHS-6040
(Rev. 2-81)

Objective:

1. To develop a membrane lung (ML) system for long term pulmonary and cardiopulmonary support.

At present, both bubble oxygenators and membrane lungs (ML) are successfully employed in brief applications during cardiac surgery requiring cardiopulmonary bypass. However, for long term application for pulmonary or cardiopulmonary assist, the ML is the only safe gas exchange device for laboratory or clinical use. It is the aim of this program to establish a safe perfusion system for long term applications.

2. To develop an artificial lung system based on a ML for long term carbon dioxide removal.

Although blood oxygenation is frequently the critical life threatening element requiring heroic measures, little is appreciated that carbon dioxide elimination is closely tied to the mechanics of breathing, the work of breathing, and to ventilator settings in patients requiring mechanical ventilation.

The extracorporeal perfusion system can be tailored to either the need of blood oxygenation requiring extracorporeal blood flows approaching that of the resting cardiac output; or to extracorporeal CO_2 removal, the latter function accomplished at an extracorporeal blood flow of but a fraction of what is required to meet body total oxygen needs. For example, resting body carbon dioxide production can be eliminated by an efficient membrane lung (because of this specific function, it has also at times been called the carbon dioxide membrane lung = CDML) at a blood flow of around 1 l/min. Such a blood flow is only 3-4 times more than during hemodialysis for renal failure; the procedure can be done in a vein to vein bypass mode; and only room air (and no dialysate) are needed for the effective removal of carbon dioxide from blood.

Hence, the use of a CDML can provide us with a tool to control mechanical ventilation, and spontaneous breathing, at will. By removing any desired fraction of metabolically produced CO_2 by a CDML, there can be a marked reduction of pulmonary ventilation up to and including total apnea. Put differently, whenever the rate of CO_2 production equals CO_2 elimination by the CDML, breathing for the purpose of maintaining blood $PaCO_2$ normal is no longer necessary. It hence can be possible to tailor pulmonary ventilation to conditions most suitable to retain the health of the lungs, to promote healing of those lungs diseased by prior processes and treatments.

Specifically, it is the aim of this program to restore health from

acute respiratory failure of any cause, including the shock lung, the perfusion lung, in lung burns, viral-bacterial-toxic involvement of lung parenchyma, etc. The common approach is to stop the previous pulmonary treatment and to resort to low frequency positive pressure ventilation at low pressures, and with extracorporeal CO_2 removal. It is likely that preventive treatment (at a much lower extracorporeal blood flow rate) may become an equally important concept in the preventing of acute lung failure from developing in the first place.

In newborns, respiratory problems are a major cause of morbidity and mortality. Apparently, the underlying mechanism of pulmonary disease is prematurity of the lungs, in contrast to that found in patients with ARF. In this case, the pulmonary ventilation (spontaneous or mechanical) can be substantially decreased if a significant fraction of metabolically produced CO_2 can be continuously removed by a CDML.

The mechanism of lung failure in chronic lung disease is of an entirely different nature. Nevertheless, it is probable that the restoration of normal arterial blood gases in these patients can help restore some significant pulmonary function, and a relative restoration of health - not unlike what is seen in chronic renal patients on hemodialysis.

3. Acute respiratory failure: adult respiratory distress syndrome (ARDS), and hyaline membrane disease (HMD).

The current outlook of patients with ARDS is poor. The results of the controlled study on extracorporeal membrane lung blood oxygenation (ECMO) for the treatment of patients with ARDS was disappointing, as no benefit could be ascribed to membrane lung use when combined with best conventional care using mechanical ventilators. It is even more surprising that the prolonged use of mechanical ventilators in critically diseased patients can lead to other organ function deterioration, so that the long term outlook of any patient requiring mechanical ventilation with an FIO_2 over 0.50 is not good. We believe that the combined effect of barotrauma and renal and hepatic dysfunction (the latter directly related to the side effects of mechanical ventilation) create a combination of factors the outlook of which becomes poor.

We subscribe to the view that ARDS is a disease not of a unique viral, bacterial, or toxic nature, but one that can recover in time. Witness the recovery from local pulmonary lesions of much greater magnitude, without aggresive mechanical ventilation being employed (there being "no need" to ventilate the patient). It is likely, that ARDS in part can develop not so much from an overwhelming general involvement of the lungs but rather, a combination of aggressive treatment with a mechanical

ventilators evolves into a lethal disease. This recognition in the past was likely masked by lack of any alternatives other than to manage lungs with ARDS by mechanical ventilation.

In the management of acute respiratory failure in newborns (hyaline membrane disease = HMD), there has been significant progress in the last dozen years through the application of continuous positive airway pressure (CPAP). This treatment is not absolute, and can lead to CO_2 retention. The outlook of patients requiring mechanical ventilation (due to CO_2 retention) is poor. It is felt that HMD represents immaturity in the pulmonary surfactant system. Hence, it was reasoned, the introduction of pulmonary surfactant (endogenous, or synthetic) was believed to represent the biochemical mode to the prevention and treatment of HMD. Unfortunately, this has not turned out to be the case.

In addition, HMD usually evolves gradually throughout the first day of life. There is moreover the problem of initial insufflation of the lungs, especially in some newborns. Those preterm newborns have sufficiently developed pulmonary vascular capillarity capable of oxygen transport; however, the terminal airways tend to be unstable due to a presumed lack of pulmonary surfactant.

We believe that lungs of preterm newborns can be managed so as to insufflate the terminal airways structures, and the acquisition of terminal airways stability without the development of pulmonary disease during the newborn period.

Methods Employed and Major Findings:

1. Within limits, any membrane lung design can be upgraded to perform well as a CDML. The key change in membrane lung conversion to a CDML involves either a prolongation of the blood path length, or the reduction of blood flow (with some recirculation, to assure uniform blood distribution). The preferred membrane material has been silicone rubber. Some microporous polypropylene membranes are now available which promise to have an advantage over silicone rubber in terms of CO_2 transfer. The major disadvantages of these microporous polypropylene membranes is their large water vapor transfer rate. In addition, it appears that some water can pass through micropores, in effect raising the diffusion resistance for CO_2 transfer, with at times disastrous fall in CO_2 transfer rate.

We have utilized microprous polypropylene capillaries to fabricate high efficiency capillary CDML of 0.1 m^2 surface area. There was no condensation of water vapor on the surface of the membrane at transmembrane pressures of less than 200 torr. There was substantial water vapor condensation when the transmembrane pressure reached 500 torr, in line

with the caution voiced by the manufacturer of the membrane.

Because of the need to ventilate tight fiber bundles is rather formidable we have devised a vacuum oscillation method to intermittently oscillate the pressure within the gas compartment at between 200 and zero torr below ambient. This results in an excellent washout of CO_2 during each and every cycle of the gas flow, and it enhances CO_2 transfer, well in excess of 100 ml m^{-2} min^{-1}.

We have no experience in long term use of microporous polypropylene capillaries for CO_2 removal. Hence, the long range outlook for microporous polypropylene membraves depends on data shwoing continuous perfromance over many days or weeks, and reliability for CO_2 removal. Such data are still lacking.

2a. Fetal Work

The fetal lamb is the animal of choice to explore newer approaches to the treatment and the prevention of HMD. The preterm fetal lamb of 130 d. gestational age has a mortality near 100%, all related to pulmonary failure. Under general anesthesia, we delivered fetal lambs of 131-134 d. gestation but left the umbilical circulation intact to effect CO_2 removal, and to add some oxygen transport. We inflated the lungs to a pressure of 15 cm H_2O (apneic oxygenation), until the total static lung compliance exceeded 0.6 ml(cm $H_2O)^{-1}kg^{-1}$. This procedure assured a good arterial blood oxygen tension, while any effect on PCO_2 was small even when the umbilical blood flow was reduced.

We have found that the total lung compliance will gradually rise over a period of a mean of 6 hours, following which the fetuses were delivered and then maintained on mechanical ventilation for 24 hours, with excellent results.

When the lungs were first insufflated to a pressure of 35 cm H_2O for 5 seconds, four times in a row, and then repeating same insufflation every 1/2 hour, the time to reach the compliance endpoint was reduced to but 1 hour. These studies showed the importance of initial pulmonary insufflation in the sequence leading to lung aeration; and more importantly, to the rise in lung compliance.

The rise in pulmonary compliance to levels stated has given us confidence that subsequent mechanical ventilation will be uneventful, and that survival be assured. We have recently explored the predictive merit of pulmonary oxygen transport to establish another end point following which the lungs can be ventilated, with survival assured. It must be recalled, that fetal circulation differs markedly from the adult blood circulation, as the fetal blood flows is diverted from the pulmonary

circulation. We felt the diversion of blood flow through the lungs (and hence resulting in an increase in arterial blood PO_2) may be of predictive value when pulmonary ventilation becomes possible. We infused acetylcholine (80 ug min^{-1}) into the pulmonary circulation and found that pulmonary oxygen uptake increased to 7-8 ml $kg^{-1}min^{-1}$ within 30 minutes. At that point, the umbilical circulation was tied, and the animals were placed on mechanical ventilation, with excellent pulmonary function following 24 hours of mechanical ventilation.

We additionally found no change in the minimum surface tension of saline lung wash in those animals, all of which had survived 24 hours of mechanical ventilation in good heatlh. This suggests that the main result of our efforts at managing the lungs was in the acquisition of normal lung compliance, the reduction in the pulmonary vascular resistance, with diversion of pulmonary blood flow so as to assume an adult cirulatory pattern. These lungs could now be ventilated, the blood PCO_2 and PO_2 were normal for newborns, with no further difficulties in sight. While the L/S (lecithin/sphingomyelin) ratio of amniotic fluid and lung fluid was highly abnormal (less than 1.0), the L/S ratio of the saline lung wash following 24 hours of mechanical ventilation was in the high normal range, but not sufficiently so in absolute quantity of surface active material to reflect in a lowering in the abnormal minimum surface tension values of the saline lung wash.

These studies showed that the surfactant system was activated, a factor necessary for the long term stability of the terminal airways system.

Our laboratory findings lead us to believe that the great majority of newborns at high risk of developing HMD can benefit from the procedures as outlined. The initial intensive pulmonary insufflation is in itself the simplest and most rapid procedure. We have now established its merit in this preterm fetal lamb population. The pulmonary insufflation combined with apneic oxygenation, and acetylcholine administration, are procedures that may well require the use of a extracorporeal CDML to safely permit the execution of this pulmonary "conditioning". We feel that such a treatment could be safely performed in a matter of an hour, if not in minutes.

b. Treatment of ARDS

These clinical studies were carried out in collaboration with the University of Milan.

A total of 11 patients were treated with extracorporeal CO_2 removal and LFPPV for ARDS. All patients met ECMO entry criteria (delineating a patient populatin with a 90% mortality rate). Within less than 1 day and

more often within 12 hours, the PaO_2 rose from less than 50 torr to over 100 torr, while the FIO_2 was lowered to 0.6. This change signified a marked improvement in pulmonary function with a reduction of QVA/Q ratio. However, improvement in lung compliance was much more slow, requiring many days, and was in part related to the duration and the intensity of the previous mechanical ventilation. Of the 11 patients, 9 patients had significant improvement in lung function, and 7 patients were eventually discharged and are at home and well.

What is significant to this new treatment is the rapidity of improvement once on bypass, and once removed from aggressive treatment with a mechanical ventilator. It is thus likely, that partial CO_2 removal with a CDML at a much earlier stage can prevent the progression of the disease process to the stage where total CO_2 support may become necessary.

Significance to Biomedical Research and the Program of the Institute:

1. Acute pulmonary problems are a serious complication in any hospital setting, whether a primary cause of hospital admission, a complication resulting from being bedridden, or postoperatively, or whether it is encountered in the immediate newborn period. It is usually a self limiting disease, with full recovery the hoped for end result, but must be managed intensively, with understanding of the many ramifications.

We believe control of ventilation by $ECCO_2R$ is an important contribution to understanding the proper management of acutely diseased lungs. This management, we feel, is tailored to immediate discontinuing of the aggressive mechanical pulmonary ventilation as now practiced, which we feel has contributed to the evolution of the problem. We needed a better understanding of the disease process, not better mechanical ventilators. The use of the CDML (just as hemodialysis for acute renal failure) is likely to emerge as a powerful tool in the scientific management of acute pulmonary insufficiency.

2. Our results from preterm fetal studies lead us to believe that newborns at high risk of developing HMD can now be managed with a much better prognosis. The clinical entity of BPD (bronchopulmonary dysplasia) attests to the serious long term consequence of present day aggressive treatment of HMD in some of today's survivors. We believe assessment in lung compliance, insufflation of the lungs, can suggest the therapeutic application of CDML with $ECCO_2R$ to condition the lungs sufficient for normal pulmonary ventilation. Such an approach may be safer than the administration of glucocorticoids to pregnant women at high risk of delivering newborns prone to develop HMD, the long term result of which on the newborn not be known for many years.

Proposed Course:

1. Studies in the application of extracorporeal CDML in patients with ARDS will continue at the University of Milan, under the immediate direction of Dr. Gattinoni. The clinical application of CDML to the treatment of HMD will be directed by Dr. Pesenti, while Dr. Solca will supervise the preventive treatment in newborn lungs at high risk of developing HMD.

2. Laboratory studies will continue in a yet younger fetal lamb population to explore the merit of apneic oxygenation and extracorporeal CO_2 removal. The perfusion system and the hardware will be simplified and made more compact so as to allow more rapid institution of bypass, and on line monitoring of lung improvement.

3. We will explore safe means of applying total (and partial) cardiopulmonary bypass to a severely failing heart. Such studies will include exploring the safe management of the failing heart (analogous to the safe management of failing lungs) to provide conditions conducive to the healing of the injured heart. Proper instrumentation will be developed to allow rapid institution of this new therapeutic modality.

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5. Gattinoni, L., Pesenti, A., Pelizzola, A., Caspani, M. L., Iapichino, G., Agostoni, A., Damia, G., Kolobow, T.: Reversal of terminal acute respiratory failure by low frequency positive pressure ventilation with extracorporeal removal of CO_2 (LFPPV-$ECCO_2R$). Trans. American Soc. Artificial Int. Organs, 1981, in press.

6. Suaudeau, J. and Kolobow, T.: Silica-Free Silicone Rubber in Isolated Heart Perfusion with Blood at 13°C. Cryobiology 18, 258-270 (1981).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01405-07 LTD

PERIOD COVERED October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Analysis of Microcirculation by Coherent Light Scattering

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

PI:	R. L. Bowman	Chief, Lab. Tech. Development	LTD	NHLBI
	P. D. Bowen	Biologist	LTD	NHLBI
Other:	R. Bonner	Physicist	BEI	DRS

COOPERATING UNITS (if any)

Biomedical Engineering and Instrumentation Branch, DRS

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 3	PROFESSIONAL: 2	OTHER: 1

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The purpose of this project is the development of a clinical, non-invasive monitor of tissue blood flow by analysis of the spectrum of Doppler scattered laser light. The NIH Laser Doppler Blood Flow Monitor has been demonstrated to be highly portable and clinically convenient with the new flexible 4m fiber optic probes and photodiode detection system. The probes withstand sterilization procedures and mechanical insult well and are suitable for operating room patient study. The linearity of the flow analysis processor has been demonstrated in a variety of tissues and clearly resolves physiologic flow changes including instantaneous pulsatile flow in the microcirculation. Muscle blood flow in over 50 patients with neuromuscular disease has been studied and preliminary data suggest that post occlusive reactive hyperemia responses may be primary or secondary indicators of disease state. Studies of scleroderma patients' skin blood flow have shown markedly reduced flows in advanced scleroderma with tremendous flows at teleangiectasia. In the general program of developing clinical applications for the instrument are; allergy testing - quantitative methodology, periocular blood flow as indicator of external-internal carotid artery flow and implied flow to circle of Willis.

PHS-6040
(Rev. 2-81)

Objectives:

To continue development of techniques for clinical and research applications of the NIH Laser Doppler Blood Flow Monitor. The linearity of the system to blood flow has been demonstrated in skin and a variety of other tissues. On going clinical applications include, muscle blood flow at open muscle biopsy in muscular dystrophy patients, skin blood flow in normals and scleroderma patients and potential for therapy assessment, skin blood flow in periocular and facial regions of patients with carotid artery occlusive disease as potential alternative to angiography, and allergy testing using quantitative skin flow responses. Specific objective at this stage is the application of the instrument and technique to a variety of clinical and experimental problems.

Methods:

The present form of the apparatus has demonstrated its clinical convenience and portability and has opened the door to many new clinical and experimental applications. The flexible fiber optic probe system has been the key development which has made the instrument portable and convenient to clinicians, patients and operator. Flexible attachment to the subject opens horizons for many new studies like exercise hypermia in skin, and chronic monitoring of flow in tissues of laboratory animals. The laser diode is to be investigated as an alternative to the present He. Ne. laser.

Acute needle probes are presently being developed for use measuring flow in human muscle and potential internal organ flow measurement which can be utilized in clean outpatient facilities.

Major Findings:

I. The fiber optic probe system greatly improved the convenience of remote and flexible attachment to the patient. We have found that the probe is extremely durable and has been utilized in operating room conditions greater than twenty (20X) applications. The probes tolerated repeated gas sterilizations and mechanical insult by operation room personnel.

II.The mean frequency detection used to analyze the Doppler shifted light signal has proved to be the optimum analysis method for diffuse tissue scattering. We have found that output flow levels ($\bar{\omega}$) in a given tissue as well as between tissues correlate well with alternative measures of flow cited in literature. Our ω output corresponds $^{\omega}$= 1 volt = 10 ml/min/100g tissue. Interacting with other researchers we have been insistent on the correctness of our processor algorithm and are actively communicating with commercial developers to insure that the proper analysis scheme is employed in the

commercial version of this instrument.

III. We have found that normalizing our flow parameter to the power of light back scattered from tissue does indeed make the device insensitive to changes in tissue color, thus facilitating comparisons of flow values from tissue to tissue as well as from individual to individual (especially where skin pigmentation is different).

IV. Studies of human tissue blood flow have been conducted under several protocols at the Clinical Center and other locations.

In addition to measurements of skin blood flow in clinical center patients and normals we have made extensive measurements of human muscle blood flow in over 50 patients during open muscle biopsy. Resting flows and postocclusive reactive hyperemia were monitored. Preliminary data analysis suggest that there are flow levels and responses which may be primary or secondary indicators of the various muscle "organ" disease state.

We have also made measurements of skin blood flow in scleroderma patients at the Water Reed Army Medical Center and have found significant reduction in finger skin blood flow in these patients. Their responses to heat, cold and occlusion are also reduced as compared to normals. Teleangiectasia exhibit flows which exceed resting flow values of normal human finger skin.

We have made measurements of special clinical center patients and normals in the periorbital region of facial skin and have found differences in flow and response to carotid occlusion for the patients not found in normals. This is especially interesting because our experiments indicated abnormal zygomaticorbital skin flow in one patient whose retinal arteries were becoming occluded with clots. No other diagnostic used for this patient showed any significant difference from normals. We feel that this finding brings the instrument closer to reaching one of the original goals of this project. This goal was to be able to infer external carotid and internal carotid artery blood flow and implied flow to the circle of Willis and brain.

We have initiated studies of blood flow and local contractility of epicardium and endocardium of the dog heart. Preliminary studies have shown that the complex wave form obtained in beating heart muscle can be analyzed as to separate contractile and flow curves by averaging over several cardiac cycles and subtracting a no-flow, contraction only signal from the combined signal. We are planning further experimentation in collaboration with Dr. Randy Patterson, NHLBI, Cardiology.

Proposed Course:

1. Continue development of the instrument in collaboration with BEIB

and industry.

2. Cooperate in clinical trials to establish the instrument as a useful clinical and experimental tool.

Significance to Biomedical Research and the Program of the Institute:

The NIH Laser Doppler Blood Flow Monitor is an instrument which holds much promise for study of the local tissue microcirculation.

It has potential applications not only in the research laboratory, but in the clinical study of vascular disease, peripheral vascular disease, allergy-skin flow testing, screening of vasoactive drugs, and the monitoring of patients with unstable circulatory systems.

Publications:

1. Bonner, R. F., Bowen, P. D., Clem, T. R., Nossal, R.: Laser Doppler Continuous Real Time Monitor of Pulsatile and Mean Blood Flow in Tissue Microcirculation Scattering Techniques Applied to Supramolecular Non-equilibrium Systems, Plenum press, 1981, p. 279-316.

2. Bonner, R. F. Nossal, R. : A Model for Laser Doppler Measurements of Blood Flow in Tissue, Applied Optics, 20: 2097-2108, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01407-18 LTD

PERIOD COVERED

October 1, 1980 through September 30, 1981

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

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.6	PROFESSIONAL: 0.6	OTHER:

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

1. A new fluorometric assay for phospholipase activity has been used. It was found that bovine serum albumin activates and inhibits phospholipases from different sources. The optimal conditions for the assay have been investigated. The mechanism of the serum albumin effects have also been studied. Albumin may interact with the enzyme, or with the substrate, to modulate the rate of liposome lysis by phospholipases.

2. Energy transfer studies on human serum albumin have been performed to estimate intramolecular distances between various binding sites on the protein.

3. Stopped-flow fluorescence measurements on the rate of labeling of proteins show that dyes attach in the millisecond range. The rate of acetylation of proteins was also studied with fluorogenic reagents.

PHS-6040
(Rev. 2-81)

Objectives:

The purpose of the project is to test new methods of fluorescence assay by applying them to problems of current biochemical interest. In this way, the methods are refined and their usefulness is demonstrated.

Methods Employed:

Chemicals were obtained commercially and tested for purity by standard methods. Fluorescence measurements of quantum yield, lifetime, spectra, and polarization were performed on modified commercial instruments. Stopped-flow kinetics were analyzed with instrumentation from this laboratory which has been described previously in conjunction with Dr. Berger. Data reduction and some figures for publication were done on the N.I.H. DEC-system 10 computer.

Major Findings:

1. Phospholipases have been studied with a new fluorometric assay for activity. The method uses phosphatidylcholine liposomes which contain concentrated 6-carboxyfluorescein, a fluorescent dye. When phospholipases lyse these liposomes, the fluorescence is enhanced as the concentrated dye is released into the surrounding medium. When present within the liposomes as a concentrated solution, the dye is virtually nonfluorescent due to "concentration quenching". We have studied this phenomenon by absorption spectroscopy and fluorescence lifetime measurements of dye at various concentrations and conclude that concentration quenching is due to dye aggregation and energy transfer to non-fluorescent aggregates.

In a study now submitted for publication, we found that BSA (bovine serum albumin) has marked effects on various phospholipases, and this modulation of activity by albumin is probably physiologically significant. BSA activated the phospholipases A_2 of snakes and bee venom, while it inhibited that of pancreas. BSA also inhibited the phospholipases C of C. welchii and B. cereus. Palmitic acid-saturated BSA failed to activate the snake enzymes, showing that the fatty acid binding site was important. However, the palmitic acid-BSA complex was a strong activator of the bee venom enzyme, probably because the fatty acid itself was an activator. Substrates which were suitable for the phospholipase assay included liposomes made from egg and soybean lecithin, as well as from pure synthetic phosphatidylcholines. Substrates in either the liquid crystalline or gel states were lysed in the assay.

The activation and inhibition of phospholipases by serum albumin may have arisen through various evolutionary adaptations, since there are certain advantages to these effects.

Other aspects of this study are being extended. The mechanism of the activation and inhibition is not clear, but from polarization of fluorescence measurements using dye-labeled phospholipases, we can detect interactions with BSA and with phospholipid substrates. Also, the fluorescence assay seems well suited to rapid kinetic studies, and we have made preliminary observations of phospholipase activity in the millisecond range. Such studies are not possible with standard titrimetic or radioisotopic assays.

2. Mapping experiments on human serum albumin, as well as albumins of other species, have been performed using the technique of fluorescence energy transfer. Previously, we estimated the distance from the single tryptophan to the single sulfhydryl group of human albumin to be about 24 A. We have extended these observations by using a variety of energy acceptor dyes attached to the sulfhydryl, including pyrene methyliodide, iodoacetamido fluorescein, iodoaminoethyl DNS, and others. Since these dyes act as acceptors of energy transfer from tryptophan, they should all yield the same tryptophan to sulfhydryl distance. The data so far seem to indicate this is so, thus ruling out nonrandom orientational artifacts. These dyes themselves are fluorescent, so they can in turn be energy donors to bilirubin or an energy acceptor bound elsewhere on the molecule. Data have so fare been accumulated in energy transfer distances as follows:

1. Tryptophan to dye attached to sulfhydryl group
2. Sulfhydryl group dye to bilirubin
3. Sulfhydryl group dye to anthroylstearic acid bound to fatty acid binding site.

When the calculations have been done we will have information on the relative distances between four sites on serum albumin. These studies have so far used mainly human serum albumin, which has but 1 tryptophan. Data have been accumulated for bovine serum albumin and others which have more than 1 tryptophan. Here the analysis will depend upon the resolution of the fluorescence decay of tryptophan into multiple components.

3. Work has continued on stopped-flow studies of dye labeling kinetics in proteins. Very little is known of the rate of labeling of proteins by dyes, because of the lack of a suitable, measurable parameter which is proportional to labeling. Most labeling reagents are colored or fluorescent before and after attachment to proteins, so the degree of labeling cannot be assessed until separation of the protein from the free dye. We have approached this problem by using a reagent, o-phthalaldehyde, which is soluble in water and nonfluorescent until it reacts with primary amino groups. So far we have collected data showing that various proteins are labeled in the range of milliseconds to seconds, and that various ligands on proteins alter the rate. The rates can be compared with the

labeling rates of free amino acids, which we previously measured in collaboration with E. Trepman. The labeling of several proteins seems to be faster than what one would expect, probably because binding of the reagent facilitates the subsequent labeling reaction.

4. Stopped-flow fluorescence has also been used to follow the acetylation of serum albumin. This phenomenon has clinical significance, because acetylation by substances like aspirin occurs physiologically. Indeed, it has been postulated that aspirin inhibits prostaglandin synthesis by acetylating a key enzyme needed in the prostaglandin pathway. Rapid acetylation of serum albumin by p-nitrophenylacetate has been studied by others, and we have discovered that the fluorogenic compound 4-methylumbelliferone acetate also will acetylate albumin. The acetylation rate is followed by production of the fluorescent 4-methylumbelliferone. Aspirin is acetyl salicylic acid, which is weakly fluorescent; but the hydrolysis product, salicylic acid, is highly fluorescent. We thus can follow directly the rate of acetylation by aspirin or other acetylating agents using stopped-flow fluorometry. Our data so far show that acetylation occurs in the millisecond to second range depending on factors such as pH, concentration of the reactants, and ligands associated with the albumin. The sensitivity of the fluorometric system allows measurements with concentrations of acetylating compounds which might obtain in vivo.

5. We have collaborated briefly with A. Moran, LKE, NHLBI in a project to study, by stopped-flow fluorescence, the glucose-dependent sodium transport system of rabbit kidney vesicles. Using a fluorescent probe, ANS, Dr. Moran has found that activation of the transport system by D-glucose is accompanied by characteristic changes in ANS fluorescence, while L-glucose fails to activate the system or cause the fluorescence changes. Stopped-flow fluorescence studies have characterized the rates of these processes.

Proposed Course:

Much of the work described above has either been prepared for publication or is nearly ready to be written up, and high priority will be given to completion of the publication process. We intend to continue to apply specialized fluorescence methods to these important biomedical problems.

Publications:

1. E. Trepman and R. F. Chen: "Fluorescence Stopped-Flow Study of the o-Phthaldialdehyde Reaction", Arch. Biochemistry and Biophysics 204: 524-532, 1980.

2. R. G. Meeks, D. Zaharevitz, and R. F. Chen: "Membrane Effects of Retinoids: Possible Correlation with Toxicity", Arch. Biochem. Biophysics 207: 141-147, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 HL 01408-16 LTD

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Methodology in Fluorescence MeasurementsResearch

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

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

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)

1. A method for assaying surface-active peptides was developed. Phosphatidylcholine liposomes are prepared containing concentrated 6-carboxyfluorescein. When these liposomes are exposed to surface-active peptides, fluorescence is enhanced in proportion to the amount of peptide. The sensitivity is high; 50 picomoles of mellitin can be detected. The assay system was tested with fractions from HPLC runs on extracts of insect venoms.

2. Fluorescence polarizaiton measurements of intrinsic protein emission have been made which show that rotational motion of the protein and of the individual tryptophan residues can be resolved. Calculations have been made to define conditions where intrinsic fluorescence is useful for measuring protein relaxation times.

PHS-6040
(Rev. 2-81)

Objectives:

The purpose of the project is to develop new methods of fluorescence assay useful in biomedical research. Attention also is given to purity, standards, and other aspects of fluorometry.

Methods:

The instrumentation used for fluorescence measurements has been obtained commercially and modified as described in the past in this laboratory. Chemical substances are obtained commercially or supplied by collaborators. The experiments do not employ human subjects.

Major Findings:

1. Liposomes which contain a fluorescent dye have been used as a basis for a new method for assaying surface-active peptides. In collaboration with Dr. J. Pisano and others in his laboratory working on pharmacologically active peptides from insect venoms, we have demonstrated that certain peptides will lyse liposomes which contain a concentration-quenched fluorescent dye, 6-carboxyfluorescein. When exposed to these peptides, the liposomes, which are about 300 A in diameter, release the dye, and the resulting increase in fluorescence is proportional to the amount of surface-active peptide present. Existing methods for measuring these peptides are time-consuming and cumbersome, involving a bioassay of activity in degranulating mast cells. The new assay is well suited to determination of peptide concentration in chromatography effluents and could be adapted to continuous flow applications.

The method was first tried on various concentrations of the bee venom peptide, mellitin. The sensitivity was such that 50 picomoles of mellitin resulted in a signal-to-noise ratio of greater than 2. The sensitivity towards the wasp venom peptide mastoporin was similar, but since the molecular weight of mastoporin is about half that of mellitin, the assay can detect a smaller mass of mastoporin. The assay was used to show activity in fractions collected by HPLC. This new method should have wide applicability.

2. Further studies have been performed on the usefulness of the intrinsic fluorescence polarization of proteins as a parameter for monitoring conformational changes. Most workers have assumed that the lifetime of intrinsic fluorescence due to tryptophan residues is too short (2-5 nanoseconds) relative to the slow rotational relaxation rate of proteins, and have therefore labeled proteins with dyes in order to follow rotational motion by fluorescence polarization methods. The region where the lifetime becomes "too short" or "too long" to be useful relative to a

given rotational relaxation rate has never been defined, so we have made calculations on this problem. It is clearly necessary to define the accuracy with which polarization can be measured, and we have been able to calculate maximum and minimum lifetime-to-rotation ratios for various experimental precisions and maximum polarizations P_0.

Another aspect of intrinsic fluorescence is the recent claim that tryptophan in some proteins rotates relatively freely in a hydrophobic "pocket", and thus is characterized by two relaxation times reflecting the motion of the protein as a whole and a more rapid independent motion. If the claim is correct, isothermal Perrin plots obtained by polarization measurements in solutions of different viscosities should reveal the protein relaxation rate, while similar measurements at increasing temperatures should reflect increasing motion of both the protein and the individual tryptophasn. We have made measurements of polarization of a large number of proteins under isothermal and non-isothermal conditions and tentatively conclude that tryptophan mobility varies from negligible to high, depending on the protein. Of interest is the finding that relaxations can be calculated for even the largest proteins studied. In the large proteins, the measureable relaxations clearly cannot reflect motion of the entire protein, but must be rotations of sidechains or the dye itself.

One concludes that intrinsic fluorescence polarization is more useful than generally realized and should reflect protein structural changes.

Significance to Biomedical Research and the Program of the Institute:

The Institute has consistently supported advances in fluorescence methodology, which has facilitated progress of biomedical research in many areas. The present work, which includes the development of a new assay system for membrane-active peptides and the characterization of the technique of intrinsic protein fluorescence polarizaiton, carries on this tradition.

Proposed Course:

The work on the peptide assay system is essentially complete and needs only to be prepared for publication. We also intend to extend the work on intrinsic fluorescence polarization and bring it to a successful conclusion. Part of our efforts will be directed to development of methods to follow decay kinetics in fluorescence, using a picosecond laser system (with Dr. G. Liesegang).

Publications:

1. Edelhoch, H., and Chen, R. F.: The Structural Analysis of Polypeptide and Protein Hormones by Absorption and Fluorescence Spectroscopy" in C. H. Li, Ed., Hormonal Proteins and Peptides, Vol. IX, Academic Press, New York, 1980, p. 109-173.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01411-15 LTD

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Blood Flow Measurements Using Nuclear Magnetic Resonance Techniques

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

PI:	V. Kudravcev	Electronic Engineer	LTD NHLBI
	J. Pochobradsky	Expert	LTD NHLBI
	R. L. Bowman	Chief, LTD	LTD NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The association with the Medical College of Wisconsin has been completed. They will continue with extramural support. This laboratory will continue only on model NMR-flow systems to develop electronic circuits and evaluate methods of flow measurement. A test of a system for signal enhancement utilizing an auxiliary sample with a fast relaxation time was shown to be no more effective than electrical filtering or phase correction.

PHS-6040
(Rev. 2-81)

Objectives:

This project is a part of our continuing program of development of methods of noninvasive measurement of cerebral blood flow using magnetic resonance.

Our present concern is to evaluate some signal enhancement methods and to improve our experimental laboratory system to permit observations on flowing liquids to evaluate the limits of detectability of magnetic marker related to the relaxation times of blood protons.

Project Describption:

In tag-detect method, the flowing blood is premagnetized in a strong magnetic field, tagged with altered states of magnetization by means of electromagnetic waves of radio frequency, and detected downstream with a nuclear magnetic resonance detector. The sensitivity of the detector and its apparent enhancement by use of an auxiliary sample with high relaxation rate placed in the detector coil was investigated. The second problem is that of limitations imposed by the shortness and the frequency dependence of the relaxation time of blood. We were looking for the optimum magnetic fields and geometry of the flowmeter. The third problem is the imporvement in the laboratory system to evaluate the limitations of magnetic labeling.

Methods Employed:

I. Possible enhancement of signal by an auxiliary stationary sample.

Our apparatus for magnetic tagging and detection downstream consisted of a water flow system, premagnetizer made of 2000 gauss permanent magnet and 50 ml meander shaped flat vessel, detector magnet (Alpha Scientific) giving 1700 gauss field with small 60 Hz modulation in 2 inch gap between 6 inch diameter pole pieces, a pulsed regenerative transmitter/receiver unit, amplitude and frequency modulated wave generator (Wavetek), various tagger and detector coils, oscilloscope and chart recorder.

We tested the hypothesis that a suitable auxiliary sample placed around the flow tube in the detector coil may increase the sensitivity of detection of water tagged with on-and-off premagnetization. As a suitable auxiliary sample serves a proton rich material that has the spin-lattice relaxation time considerably shorter than water. When the power of radiation from the transmitter is so high that the signal from unmoving water vanishes due to saturation, the auxiliary stationary sample still gives a signal. As a liquid auxiliary sample we used water solutions of $MnSO_4$ and as a solid sample latex rubber. We tried sundry configurations of rubber strips, sheets and tubes in detector coils of various sizes, and

also two separated coils connected in series, one with flowing water and one with the auxiliary sample. Several glass concentric double-tubes were made and used; water flowed through the inner tube and manganese sulfate solutions of various concentrations filled the outer tube.

We looked for a possible improvement of signal amplitude, signal-to-noise ratio and a possible sharpening of the resonance curve due to the filter effect of the auxiliary sample.

Major Findings:

The inserted auxiliary sample merely adds signal and slightly changes quality factor of the coil. There is no double resonance due to its direct interaction with protons of flowing water. It interacts only with the coil and has the same effect when placed in another coil separate from water. When the signal was seemingly enhanced beyond simple addition, it was due to a shift of the resonance frequency, as was shown by plotting the tuning curve with and without the auxiliary sample.

In the case of tagging with reversal instead of erasure of magnetization, the rectification of the signal makes it difficult to detect the tag. However, the auxiliary sample adds a steady signal and reveals the tag after rectification.

II. Optimum detector field.

On the basis of published frequency dependence of spin-lattice relaxation time of blood we have calculated the optimum field for detection of magnetically tagged blood. The calculation and results are described in a just finished paper.

III. Laboratory appratus improvement.

We obtained a seven inch gap permanent magnet with 16 x 17 inch pole pieces, field bias coils and power supply from the Medical College of Wisconsin. We modified it by making the gap adjustable, adding modulation coils and improving the power supply.

In cooperation with the Medical College of Wisconsin we built a 2.735 MHz transmitter, a 2.739 MHz narrow band reciever, a flat NMR crossed coil and a pulsating flow system with electornic control of pulse frequency and flow rate.

The system was tested with pulsatile flow of water doped with copper sulfate to relaxation time equal T_1 of blood in 640 gauss field, and with blood flow in the arm.

Significance to Biomedical Research and the Program of the Institute:

Proton magnetic resonance flowmetry has a promise as a harmless method fo studying cerebral circulation, detection of the early course of cerebral atherosclerosis or other changes leading to cerebral atrophy, and following the results of early therapy.

Proposed Course:

The Medical College of Wisconsin will continue the cerebral blood flow program with extramural support and our program will continue to study electronic techniques and model systems relevant to the development of systems for biological measurements.

Publications:

1. Halbach, R. E., Battocletti, J. H., Sances, A., Jr., Bowman, R. L., and Kudravcev, V.: Ranging for individual artery flow in the NMR flowmeter. IEEE 1980 Frontiers of Engineering in Health Care, Washington, D. C. Sept. 1980, pp. 356-359.

2. Battocletti, J. H., Halbach, R. E., Sances, A., Jr., Bowman, R. L., and Kudravcev, V.: Current status of NMR blood flowmeter research at MCW. Proc. 33rd Ann Conf. Eng. Med. Biol. 22: 130, Washington, D., C., October 1980.

3. Halbach, R. E., Battocletti, J. H., Sances, A., Jr., Larson, S. J., Bowman, R. L., and Kudravcev, V.: Blood flow detection using the flat crossed-coil NMR flowmeter. IEEE Trans. on Biomed. Engr., BME-28(1):40-42, Jan. 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01413-19 LTD

PERIOD COVERED October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Instrumentation for the Study of Pre-Steady State Enzyme Kinetics

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

PI:	R. L. Berger	Chief, Sect. on Biophysical Inst.	LTD	NHLBI
	P. Smith	Visiting Scientist	DRS	BEI
	G. Liesegang	Sr. Staff Fellow	LTD	NHLBI
	P. Bungay	Chemical Engineer	DRS	BEI
	M. Perrella	Professor	Univ. of Milan	
Others:	J. Froelich		N.I.A.	
	C. Gibson	Electrical Engineer	DRS	BEI
	W. Friauf	Chief, Elect. Eng. Section	DRS	BEI
	H. Cascio	Electrical Engineer	DRS	NEI

COOPERATING UNITS (if any)

Biomedical Engineering and Instrumentation Branch

LAB/BRANCH
Laboratory of Technical Development

SECTION

Section on Biophysical Instrumentation

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A thermal stopped flow investigation of the binding of Ca(+2) and Li(+) to the ionophore 211 has been made. The reaction was found to proceed via the mechanism $M^{n+} + L = (ML)_1^{N+} = (ML)_2^{n+}$. Both kinetic and thermodynamic data have been obtained for this reaction using the one instrument. Additionally this binding mechanism is representative of many enzyme-substrate reactions and suggests the wide applicability of the TSF technique to biochemistry. The binding of Ca(+2) to the widely used buffer EGTA has been investigated. The rate constant for complexation has been observed to be $10^6 M^{-1} S^{-1}$ at physiological pH. This rate is quite slow and unexpected proving quite important to the accurate use of EGTA as a calcium buffer. An improved ball mixer has been developed which reduces the pressure drop by 30% and the flow heating by a factor of ten due not only to the mixer but also to a reduction in right angle turns and an enlargement of entry ports. Trials have begun on a low temperature quenching system using this mixer and a single commercially available stepping motor dilutor. A resolution of 5 milliseconds have so far been obtained.

PHS-6040
(Rev. 2-81)

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 methods and instruments to study, in collaboration with other laboratories, the reactions of hemoglobin with the respiratory gases both in normal state as modified by the changes of physical factors, small molecules, various metabolites, and genetically, such as in sickle cell anemia. The reactions of various cellular enzymes, particularly ATPase, lactate dehydrogenase, and 2-3DPG and their interactions and control in the cell are studied as they relate to the hemoglobin reactions in cardiology, pulmonary and respiratory function, and circulation. Where appropriate analytical methods are developed for research and clinical application.

Methods Employed:

The methods used in the investigation of the mechanisms of enyzme action are those of pre-steday 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 commerical firms, special university facilities, or at several special research laboratories such as the Jet Propulsion Laboratory. In pursuing these investigaions, a wide variety of physical parameters must be studied, which leads to the need for an understanding of the underlying physical theory governing the reactions. Expert consultants and collaborators are brought in to assist in the design, analysis, and evaluation of the equipment, particularly as it is applied to certain specific enzyme systems under investigation.

Major Findings

The fast thermal stopped flow is being rebuilt to take advantage of a number of improvements we have been making in flow apparatuses. A series of ball mixers have been investigated both theoretically and experimentally to try and minimize the pressure drop and heating which is produced in these systems. An improvemnt in flow velocity of nearly 30% has been achieved in the 1.5 millimeter system. Heating has dropped nearly a factor of ten due in part to the mixer and in part to the elimination of right angle turns and the enlargement of input tubes. This has made it possible to use a commerical stepping motor digital diluter to drive the syringe. A

flow velocity of 2.2 M/sec is obtained in a 1.5 mm i.d. tube. This gives a quenching time of 5 milliseconds or better in a 0.5 mm i.d. tube. This will be used in the separation of intermediates in CO and O_2 hemoglobin.

EGTA

The kinetics of calcium ion complexation by EGTA were investigated using the stopped-flow technique. This study was performed within the pH range 5.8 to 8.4. The reaction was found to be first order in both EGTA and calcium with an observed second order rate constant (pH 6.8, T = 25°C, I = 0.1 M) of $2 \times 10^6 M^{-1}s^{-1}$. This rate constant was independent of the hydrogen ion concentration yielding a value of $4.0 \times 10^8 M^{-1}s^{-1}$ at pH 8.4. The rate constant at 16° and 38°C (pH6.8) was found to be 0.92 and 6.7 uM^{-1}, respectively.

Thermal Stopped-Flow

The recently developed thermal stopped-flow system has been employed to monitor the kinetics of Ca(2+) and Li(+) ion complexation by the 211 cryptand (4, 7, 13, 18-tetraoxa-1, 10-diazabicyclo [5,5,5,]eicosane). The experiments were carried out in aqueous media buffered at pH 11.4. The thermal stopped-flow system has a dead time of ten milliseconds with a sensitivity of two millidegrees. Both Ca(2+) and Li(+) exhibited a unimolecular reaction, after ion complexation, suggesting a possible exclusive to inclusive rearrangement of the complex. Rate constants and several thermodynamic parameters for the individual reaction steps have been measured.

Proposed Future Work

The fast thermal stopped-flow apparatus will be rebuilt into a small more compact and easier to use unit. Particular emphasis will be put upon optimization of the design for performance, ability and ease of operation by the biochemists, computer coupled control, data acquisition, correction and model testing on line. Work will continue on a new multiple mixing, continuous flow system to be used with acid and low temperature quenching and filtration. Completion of the laser flash dual wavelength system will be carried out.

Significance to Biomedical Research and the Program of the Institute

The study of the kinetics of chemical and enzyme-substrate reactions is a widely applied field yielding important information for interpreting the mode of action of the chemical or enzyme under study. Continued development of the response and the sensitivity of different sensor techniques, eg. thermal, pH, and optical methods, enhances the ability for an investigator to probe with finer detail a system of interest. The particular reactions chosen to test the capability of the instrumentation being developed are of immediate significance: binding of calcium by EGTA is important for studying muscle contraction kinetics, studying hemoglobin-ligand binding studies provide information for understanding the complex action of this molecule; the cryptand study is a fine example of a reaction which can only be observed by thermal methods.

Publications

1. Malyj, M., Smith, P.D., Balko, B., and Berger R.: Thermal kinetics using a modified commercial stopped flow apparatus. Rev. Sci. Instrum. 51 51:(7), 896-899 July, 1980.

2. Balko, B., Bowen, P., Berger, R. L., and Anderson, K.: Fast stopped-flow microcalorimeter, J. Biochem. & Biophys. Methods, 4 (1981) 1-28, 1981

3. Balko, B., Berger, R. L., Anderson, K.: Measurement and simulation of thermistor response time in the millisecond range. Rev. Sci. Instrum. 52 (6) Jun. 1981, in press.

4. Liesegang, G. W.: Kientics of Lithium (+) and Calcium (2+) complexation by 211 cryptand using a stopped-flow caloriemter. J. Amer. Chem. Soc., 103: 953-955, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01414-09 LTD

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Development of Microcalorimeters and Differential pH Thermal Titration Apparatus for Biochemical Reaction Studies

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

P.I. :	R.L. Berger	Chief, Biophys. Tnstrum. Sect.	LTD NHLBI
Others :	H. Hopkins	Prof. of Chemistry	Georgia St. U
	C. Mudd	Mechanical Eng.	BEIB DRS
	W. Friauf	Chief, Ele. Eng. Sect.	BEIB DRS

COOPERATING UNITS (if any)
Biomedical Engineering and Instrumentation Branch, NIH

LAB/BRANCH
Laboratory of Technical Development

SECTION
Section on Biophysical Instrumentation

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 3	PROFESSIONAL: 1	OTHER: 2

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The differential batch calorimeter has been undergoing extensive testing. Problems with reaction cells have been corrected and extensive work has been carried out to improve the detectors, amplifiers, controllers and computer programs. Similar efforts have been carried out on the differential-pH-Thermal titration apparatus.

PHS-6040
(Rev. 2-81)

Objectives:

An understanding of the fundamental mechanism of biological reactions requires three physical factors a) the kinetics, i.e., pathway of the chemical reaction b) the thermodynamics of the reaction, i.e., what energy is available and how does it change from one state to another and c) the stereochemistry of the reactants. This project addresses these factors by developing appropriate instrumentation to study heat changes which will give a deeper insight into the mechanism of the relevant biochemical reactions involved in the functions of enzymes and cells. Model reactions are chosen to demonstrate the efficiency of the method.

Methods Employed:

Initial designs are constructed in this laboratory with special assistance from commercial firms in the construction of sensors; contracts are let, where warranted, for the development of completed instruments with refinements that would tax our own facilities. The instrument is then tested in conjuction with other interested biochemical calorimetrists utilizing appropriate enzymatic and cellular reactions.

Major Findings:

A number of parameters have been investigated during the past year to document where significant instrument improvement could be made. New detectors have been fabricated for us by Marlow Industries and recent tests have demonstrated a three fold increase in sensitivity. This has prompted us to consider a somewhat novel design for the cell and thermopile. So far, tests indicate a very workable system which may permit us to operate the calorimeter as a super Warburg respirometer. 10^5 calories are released for each mole of oxygen consumed during fatty acid metabolism. We can detect 5×10^{-9} calories i.e., 10^{-11} moles of oxygen. While this is several orders of magnitude away from single cell capability it is still in a reasonable biological range. By controlling PCO_2 & PO_2 reactions with metabolites and drugs could be readily explored using different types of cells such as liver or epithelium. A number of improvements have been made to the pH-thermal apparatus and considerable testing of both the hardware and software are presently under way. A commercial version is presently being used by investigators to study super DNA.

Proposed Course:

A new differential calorimeter will be tested and applied to a number of biochemical reactions. In addition, an attempt will be made to operate it in a scanning mode to study the intra-molecular forces in the folding of proteins. Work will continue on the flow microcalorimeter and the Differential pH apparatus.

Significance to Biomedical Research and the Program of the Institute:

An understanding of the basic mechanisms by which enzyme catalyzed reactions carry out the vital processes of life is of fundamental importance. A necessary part of this understanding is provided by a measurement of the thermodynamics of the reaction. This will ultimately lead to enhancement of our ability

to prevent and treat the heart, lung and blood ills the Institute is charged with.

Publications:

1. Mosca, A., Dossi, G., Luzzanna, M., Rossi-Bernardi, L., Friauf, W.S., Berger, R.L., Hopkins, Jr., H.P., and Casey, V.: Improved Apparatus for the Differential Measurement of pH: Applications to the Measurement of Glucose, Anal. Biochem. in press, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01415-08 LTD

PERIOD COVERED October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Italy - U. S. Cooperative Science Program - Blood Gas Instruments, Project 78

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

	Name	Title	Affiliation	
PI:	R. L. Berger	Chief, Sect. on Biophysical Inst.	LTD	NHLBI
	L. Rossi Bernardi	Professor of Biochemistry	U. Milan	
	R. Winslow	Chief, Red Cell Disease	CDC	
	C. Monge	Senior Fogarty Scholar		FIC
	T. Clem	Electrical Eng.	BEI	DRS
Others:	L. Thiabualt	Assistant Professor	U. Pa.	
	H. Cassio	Electrical Engineer	BEI	DRS
	A. Markowitz	Electrical Engineer	BEI	DRS
	W. Mangum	Physicist	NBS	

COOPERATING UNITS (if any)

Biomedical Engineering and Instrumentation Branch
University of Milan, School of Medicine, Milan, Italy

LAB/BRANCH
Laboratory of Technical Development

SECTION
Section on Biophysical Instrumentation

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5	4	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The new OEC apparatus has been put into operation and testing of various components has progressed. A number of modifications to the cell, electrodes, and electrometer should greatly improve the performance and reliability. A new digital thermistor bridge has been constructed and is undergoing calibration, tests and evaluation in the Clinical Thermometry Section, NBS.

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

An understanding of the various physical factors that affect the delivery of oxygen to the tissues and the removal of CO_2 are vital to a correct interpretation of the physiological response of the human body to various disorders, disease states, and environmental stress. As part of a long range basic research program being carried out in collaboration with the School of Medicine, University of Milan, the Hematology Division, Bureau of Laboratories, CDC, and the Cero dePasco High Altitude Research Laboratory, Universidad Peruana Cayetacho Heredia, Lima, Peru, we are engaged in the development of highly accurate instrumentation to measure the oxygen dissociation curve in whole blood and concentrated hemoglobin solutions, the hemoglobin components, in whole blood, Hb, Hb+, HbCO, and HbO_2, the pH, pCO_2, and PO_2, and the concentration of 2-3 DPG. Ultimately we hope to measure the tissue pO_2 and PCO_2 levels at the mitochrondia cytochrome oxidase site.

Methods Employed:

A systematic analysis of the complex interrelationships among several variables and the effect on the oxygen dissociation curve requires the development of a method to obtain oxygen dissociation curves of human blood in vitro, under conditions closely simulating the in vivo situation of the patient. Instruments are developed either at NIH and/or Milan, tests on pure hemoglobin are generally conducted first in Milan, where a large group is currently working on the purification of hemoglobin.

Testing on patient blood is then carried out at the Red Cell Disease Branch, CDC and at Cero de Pasco, Peru. Close cooperation exists with the medical school hospital in Milan where on-line computer monitoring will be carried out using the membrane oxygenator system developed in this laboratory by Dr. Theordor Kolobow.

Major Findings:

A new oxygen equilibrium curve apparatus (OECA TM) cell has been constructed to accommodate greatly superior oxygen and carbon dioxide electrodes and give improved temperature control. In addition, the cell has been modified so that it can use a membrane for gas exchange thus permitting use in concentrated hemoglobin solutions as well as whole blood. This new method is under complete microprocessor control with on line data collection and analysis.

Further modification of the OEC were found necessary in order to permit easy changing of electrodes. A new system for calibration was developed and extensive component testing is presently underway to evaluate accuracy, reliability and sensitivity. New membranes for the pO_2 and

pCO_2 electrodes have been devised and appear to significantly increase the response of the pCO_2 electrode. Further tests will be needed to demonstrate this effect. A careful rewriting of the system computer program has been carried out with the resulting elimination of a number of "bugs".

An important parameter in the OEC measurement is the absolute temperature. With this in mind a new, compact, digital thermistor bridge, suitable for laboratory use, has been constructed, together with several new probes. An absolute accuracy traceable to NBS has been achieved for $\pm$ .01°C.

Significance to the Program of the Institute:

An understanding of the factors controlling the delivery of oxygen and the pickup of CO_2 in the tissues as well as their subsequent movement across the alveolar is of fundamental importance to both the prevention and of heart, lung, and blood diseases.

Proposed Course:

The diffusion OEC apparatus and the OECA TM will be carefully tested as to whether they both give the correct shape of the ODC under all conditions.

Publications:

1. Berger, R. L., Clem, T., Gibson, C., Siwek, W., and Sapoff, M.: A digitally linearized thermistor thermometer references to OPTS-68. Clin. Chem. Vol. 26, No. 13, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01421-06 LTD

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Development of Electrochemical Methods for Kidney Research and Blood Analysis

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

PI:	R. Steele	Physical Scientist	LTD	NHLBI
Others:	J. Handler	Section Chief	KE	NHLBI
	M. Burg	Laboratory Chief	KE	NHLBI
	P. Popowicz	Guest Worker	KE	NHLBI
	A. Preston	Chemist	KE	NHLBI
	W. Haller	Laboratory Chief	NBS	

COOPERATING UNITS (if any)

Laboratory of Kidney and Electrolyte Metabolism, NHLBI

LAB/BRANCH

Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1	PROFESSIONAL: 1	OTHER:

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Work on methods and apparatus for the study of kidney epithelial cells grown as sheets on porous membranes continues to yield important results. The central element is the porous bottomed culture device (PBCD) in which the membrane is cemented to polycarbonate rings which provides electrical and chemical isolation between the solutions on each side of the membrane. With these PBCDs new knowledge of the development of epithelial is being obtained. The increased exchange at the basal side (blood side) leads to a vasopressin response for A6 cells (from Xenopus laevis kidney) which is otherwise absent. Omission of all serum from the apical medium (urine side) results in a larger and more rapidly developing potential difference (PD) across the epithelia. Transparent collagen membranes on the PBCDs have allowed these devices to be used effectively for primary cultures such as for medulary thick ascending limb cells from rabbit kidney. The importance of Ca# activity in the control process of epithelial cells has resulted in a project aimed at improving the selectivity of Ca# electrodes. Improvement is particularly needed for electrodes with tip diameters <0.1 microns so that activities less than 10^{-6} molal can be measured in epithelial cells.

PHS-6040
(Rev. 2-81)

Objectives:

(1) Develop porous bottomed culture devices with special properties:

A. Transparent membranes
B. Membranes thin enough for microscopy and microelectrode work
C. Small enough for slow growing cells
D. Very small for growth from one cell
E. Ultra small for one cell only

(2) Design sterile continuous flow apparatus for study of epithelial cells layers on transparent membranes so that the development of transport and morphology can be studied for weeks or even months.

(3) Develope electrodes to measure $Ca^{\#}$ activity in epithelial cells as they develop their transport capability.

(4) Develope a differential $_{p}O_2$ electrode system for the measurement of oxygen consumption by epithelial cells growing on porous membranes.

Methods Employed and Major Findings:

Porous bottomed culture devices, PBCDs, have been constructed with 1, 5 and 6 millimeter diameter collagen membranes. These have been useful for observing cells as they grow on these transparent membranes. Cells from the kidney of the Xenopus laevis (established line A6) were placed on a 6 mm collagen membrane and have been followed for more than 8 months. The PBCD was made with rubber feet which spaced the membrane off the bottom of the dish about 2 mm. At one month, the cells had developed beating cilia. They had also developed a resonse to vasopressin which had not been seen before with the PBCDs sitting directly on the bottom of a dish.

The PBCDs allow the media and PD to be different on the 2 sides of the membrane. At present the PD across the membrane is that which the cells produce. This is similar to what would occur in the animal as it develops. Until recently the same medium (including serum additive) has been used on both sides; however, in order to control cell growth (to avoid multilayers of cells) the apical medium in some experiments has been replaced by serum free medium. This did give some growth control. In some cases it also gaves PDs which started up sooner and went higher. On collegen membrane PBCDs it could be seen that the movements and shape changes of the cells were simplified and they went more directly to the final form when the serum free medium was put on the top when the cells were first confluent. It appears that we have some very useful tools with which to study the basic developmental processes of epithelia.

Many bits of medulary thick ascending limb, MTAL, of rabbit tubules have been placed on 1 mm collagen membrane PBCDs. The cells cover the membrane in about 10 days. The majority of these produce potentials ranging from 1 to 23 millivolts, apical side positive. Placing the membrane portion of the PBCDs in medium containing rabbit and bovine serum results nearly 100% of the tubules yielding confluent cell layers rather than the 30 to 50% obtained without this treatment. Experiments have shown that these cells grown on collogen behave like the perfused MTAL in many ways. They are sensitive to Furosimide, but not to Amiloride or vasopressin (in mammalian concentration). Also the PD is positive on the apical or luman side.

The importance of calcium to the control processes of epithelial cells has caused us to start work on improving the selectivity of ion specific $Ca^{\#}$ electrodes. The present techniques for mesuring $Ca^{\#}$ activity inside cells, dyes and electrodes, both give results which are probably too high. The dyes indicate to high an activity because the ones that work at all have $Ca^{\#}$ affinities which are comparable to some of the things inside the cells which bind $Ca^{\#}$. The electrodes read too high due to interferences from other ions: Na^{+}, K^{+}, $Mg^{\#}$, etc. These interferences are particularly troublesome when the electrode tips are made 0.1 microns or less in diameter. Such tip sizes are necessary to make stable mesurements inside epithelial cells. The changes being explored to obtain improved specificity are:

(1) Use of glasses without diffusable ions such as fused quartz and Corning 1723.

(2) Silanization of the glass tips with monochlorosilanes with long hydrocarbon chains and with careful exclusion of water vapor.

(3) Explore attachement of the $Ca^{\#}$ exchange molecules directly to the glass.

It is hoped that these efforts will result in electrodes with tips smaller than 0.1 microns which can accurately measure $Ca^{\#}$ activities of less than 10^{-6} molal which probably exists in most cells.

Significance to Biomedical Research and the Program of the Institute:

The apparatus and methods necessary to grow and study sheets of epithelial cells on membranes is advancing the study of the basic mechanisms of active Na transport. The ability to make the measurements under sterile conditions greatly increases the productivity of this work. Dr. Handler, LKEM, NHLBI, is presenting the physiological significance of this work in detail. The growth of the cells on nutrient and gas

permeable membranes results in a degree of development and differentiation which does not occur on the conventional plastic or glass surface. This should allow a study of the development of epithelial that is not possible otherwise. The accurate measurement of the Ca# activity inside the epithelial cells at various stages of transport development should greatly increase our knowledge of transport development and control.

Proposed Course:

(1) Design sterile continuous flow apparatus for sterile study of sheets of epithelial cells on transparent membranes for weeks or even months so that the development of transport and morphology can be followed.

(2) Develop very small collagen PBCDs which can be seeded with only 1 cell. The feeder layer often required in cloning can be provided on the other side of the membrane. The feeder cells can grow either in the bottom of the dish holding the PBCD or right on the membrane (planted previously with PBCD upside down).

(3) Develope electrodes to measure Ca# activity in epithelial cells which have tips smaller than 0.1 microns and low interferences so that Ca# activities of 10^{-6}, 10^{-7} and even 10^{-8} molal can be measured correctly.

Publications:

1. Steele, R. E., and Gilbert, D. L.: Calcium and potassium activities in the hemolymph of the squid, Loligo pealei. Biological Bulletin 159:, 492, 1980.

2. Schwartz, G. J., Weinstein, A.M., Steele, R. E., Stenphenson, J. L., and Burg, M. B.: Carbon dioxide permeability of rabbit proximal convoluted tubules. Am. J. Physiol. 240: (Renal Fluid Electrolyte Physiol. 9) F231-F244, 1981.

3. Handler, J. S., and Steele, R. E.: Epithelial function in cultured epithelial cells. In Press. First International Workshop on Developmental Renal Physiology, 1980.

4. Johnson, J. B., Steele, R. E. Perkins, F. M., Wade, J. B., Preston, A. S., Green, S. W., and Handler, J.S.: Epithelial organization and hormone sensitivity of toad urinary bladder cells in cluture. In press, Am. J. Physiol. (Renal Fluid Electrolyte Physiol.), 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01423-05 LTD

PERIOD COVERED October 1, 1980 through September 30, 1981

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, Sect. on Biophysical Inst.	LTD	NHLBI
	B. Balko	Expert	LTD	NHLBI
	G. Liesegang	Sr. Staff Fellow	LTD	NHLBI
	P. Smith	Visiting Scientist	DRS	BEI
Others:	T. Traylor	Professor of Chemistry	UCSD	
	E. Bucci	Professor of Biochemistry	UMD	
	A. Schechter	Chief, Macromolecular Biology	LCB	A
	G. Hoy	Professor of Physics	Old Dominion U.	
	R. Hendler	Chief, Sect. Membrane Enzymology	LB	NHLBI

COOPERATING UNITS (if any)

Biomedical Engineering and Instrumentation Branch
Department of Chemistry, UCSD

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 5	PROFESSIONAL: 2.5	OTHER: 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 heme involvement in cooperativity of hemoglobin was investigated using Mössbauer spectroscopy. Strained and unstrained forms of model compounds of myoglobin and normal and modified hemoglobins enriched in ^{57}Fe were studied Low temperatures (4.5K) and strong magnetic fields (50K Gauss) were used to split the spectra and line shape theory utilizing superoperator technique was used to analyze the data. At least two different heme sites for iron were found in HbA and a new heme structure similar to that of an unstrained model comound was found in the modified Hb system with characteristics similar to Hb. Osler. Bleomycin, a glycopolypeptide with cytotoxic properties, was investigated and two nonequivalent iron sites were found. Electronic relaxation in the Mossbauer spectra differentiated the active from the inactive form. The design and construction of a picosecond laser spectroscopy system has been completed. Experiments are underway for applying this technique to a series of biochemical problems. A serious impairment to the application of a vidicon to detect transient events has been observed and well characterized. A technique has been devised which overcomes this impairment as well as significantly advancing the area of vidicon imaging to pulsed spectroscopy.

PHS-6040
(Rev. 2-81)

Methods Employed and Major Findings

Model Compounds

In an attempt to understand the heme involvement in hemoglobin cooperativity we have investigated model compounds of myoglobin provided by Professor Teddy Traylor of UCSD, in the stressed and unstressed states. We found a direct relationship between the separation of the lines in the Mössbauer spectrum and the strain induced on the proximal base in these model comounds. Furthermore by studying these compounds at temperatures from 60 mK to 78 K in a helium dilution refrigerator we found that the strained heme held the iron much more tightly as determined by the recoilless fraction measurement.

Comparing these results to the results obtained with enriched ^{57}Fe modified hemoglobin (with properties similar to hemoglobin Osler i.e. high affinity - low cooperativity) we found a striking similarity in that high affinity gave lines closer together (about 50% change) indicating less strain as in the model compounds. This correlation between the strain on the proximal base, pulling it away from the heme plane and the electronic spin and valence state of the iron ion, in both the model compounds and the modified hemoglobin, will permit us to investigate in the most detailed way the molecular-mechanical model of the heme involvement in hemoglobin cooperativity and the trigger mechanism involved in the change from tense to relaxed states.

Enriched Hb

Recently we obtained iron-57 enriched hemoglobin, prepared by chemical replacement, enhancing the signal-to noise ratio in the Mössbauer transmission experiments by a factor of 50. Performing experiments on this isotopically enriched Hb at cryogenic temperatures (5K to 150K) and in a 50K gauss magnetic field we split the two line spectrum into six lines of unequal width. This showed for the first time the existence of different iron environments in the hemoglobin tetramer. Theoretical investigations of the spectrum showed that two completely different electric field gradients could be observed in the tetramer.

Using enriched carboxy Hb of a variant type with high oxygen affinity and low cooperativity we found differences in the Mössbauer spectra between this and normal Hb. This result connects biological differences in Hb to changes in the structure of the iron heme environment which can be studied quantitatively by Mössbauer spectroscopy. Conseuqently this may give information important for the understanding of cooperativity and oxygen affinity in Hb and a basis for the understanding of diseases on a molecular level.

Bleomycin

We have been investigating the antitumor properties of bleomycin whose cytotoxicity coincides with DNA degradation when both ferrous iron and

oxygen are available. We have shown that at lower pH bleomycin has two sites for ferric iron, one of which is paramagnetic and was not observed in aramagnetic and was not observed in earlier ESR or optical experiments. As the pH is raised a diamagnetic component with a strong electric field gradient at the iron site becomes dominant. At pH 7, this disappears and a single site with a weak electric field gradient is present. When ferrous iron is introduced into bleomycin at low pH a single magnetic component appears. Further studies at higher pH and in the presence of oxygen and DNA are planned.

Picosecond Spectroscooy:

A. Optical Multichannel Analyzer

A Princeton Applied Research Model 1254B SIT Vidicon and 1216 Vidcon controller have been interfaced to a DECLAB-11/MNC computer system. Testing of the OMA revealed a non-linear vidicon output response to the incident pulsed illumination level. It was determined that the factor affecting this non-linear response was the variable recharging rate of the vidicon surface from medium to low light levels. It was also observed that all vidicon scanning parameters alter this response and a careful calibration of the vidicon must be carried out, at a particular set of scan parameters, if the OMA is to be used in pulsed spectroscopic applications.

Careful calibration of the vidicon is highly impractical and so a prepare-expose-read cathode voltage switching technique has been devised which substantially improves the output linearity of a SIT Vidicon in the pulsed illumination mode of operation. This technique now allows the quantitative useage of the vidicon in pulsed spectroscopy. This technique is now being incorporated onto all PAR OMA systems as well as numerous spectroscopic systems.

B. The major emphasis of the picosecond laser spectroscopy project has been directed toward obtaining a complete understanding of the vidicon detector output. That this is of prime importance to the project cannot be overstated when it is considered that results obtained otherwise would not stand scrutiny and be qualitative at best. As is outlined above a signficant contribution has been made to the vidicon detector field in this regard.

The picosecond laser system is now fully operational. Overlap of the time-dispersed continum from the echelle and the 530 nm photolysis beam has been established using a carbon disulphide cell and the zero time segment identified. These segments are well resolved on the vidicon. The response of the spectrometer is currently being investigated by studying the known relaxation of the dye azulene;the characteristics of other dyes, not previously studied are being investigated. Electronic synchronization of the firing of laser system at the end of the last prep frame of the vidicon was achieved using an electronic circuit which features opto-isolators.

These protect the vidicon controller and data acquisition system from the rapid transients associated with the laser firing and pulse selector. The cyrogenic system has been received and a sample holder has been manufactured for this unit.

Proposed Future Research

1. Measurements on the ground state repopulation times of several organic dyes will be measured to provide information on the limits of detection sensitivity for the absorption picosecond spectrometer.

2. The ground state repopulation times (being a function of the solvent) for several dyes in membranes will be investigated to provide an understanding of the role of membrane dynamics to cellular function.

3. Photolysis experiments on model heme proteins, myoglobin and hemoglobin will be performed to delineate the binding, cooperativity and the "trigger" mechanism of hemoglobin.

4. The primary events in the UV sensitization and destruction fo certain proteins and enzymes will be studied with this technique.

5. Development of a picosecond laser spectroscopy system to probe the Raman scattering of biomolecules. This technqiue will provide an explicit probe of molecular motion and be used to expand the relationship of rapid molecular motion to catalytic activity.

Significant to Biomedical Research and the Program of the Institute

The detailed dynamics of molecular interactions between parts of the protein molecule during ligand reaction is needed for the understanding of the mechanism of all protein reactions. Thus the Mössbauer and picosecond research instrumentation hold the promise of greatly extending our understanding of the mechanisms by which the hemeproteins, such as hemoglobin and the cytochromes react. This is of fundamental importance for the understanding of and the prevention and treatment of heart, lung, and blood diseases.

Publications:

1. Balko, B., and Hoy, G. R.: Selective Excitation Double Mössbauer Spectroscopy, in Mössbauer Spectroscopy: Applications in physics, chemistry and biology, ed. B. V. Thosar and P. K. Iyengar, Elsevier, Amsterdam, 1980.

2. Balko, B., Berger, R. L., and Anderson, K.: Measurement and simulation of thermistor response time in the millisecond range, Rev. Sci. Instru. (in press).

3. Liesegang, G. W., and Smith, P.D.: A technique for improving vidicon (OMA) linearity in the pulsed illumination mode. Applied Optics, (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01434-03

PERIOD COVERED October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Microcolorimetric Measurement of Magnesium Ions

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

PI:	G. G. Vurek	Senior Investigator	LTD NHLBI
	M. Knepper	Staff Associate	K&E NHLBI
Others:	J. Bordeau	Research Physiologist	V.A. Hospital

COOPERATING UNITS (if any)

None

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Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Measurement of picomole amounts of magnesium, phosphate, and calcium contained in nanoliter volumes of biologic fluid has been accomplished with a new microcolorimeter. The colorimeter has a working volume of 220 nL, and is part of a continuous-flow analysis system. The sensitivity of the technique is better than 10^{-13} moles for calcium.

PHS-6040
(Rev. 2-81)

Project Description

Objectives:

The objective of this project was to explore new designs for flow-through colorimetric instruments and demonstrate their utility for the measurement of biologically significant ions and other materials.

Methods Employed:

The fundamental innovation in this project was the development of the flow-through cuvet made of a single piece of glass tubing with blown-end windows. This cuvet has a working volume of 220 nL and an optical path of 1 cm. This cuvet is part of a continuous-flow analysis scheme. Reagent, specific for the material of interest is drawn past the injection port where samples are placed. The samples react to form a colored product, the optical transmittance of which is measured on a solid-state photometer containing the cuvet. Sub-picomole sensitivity is provided by the small cuvet volume and the reagents employed provide the specificity required.

Major Findings:

Three separate colorimeter systems have been built and tested. The first, using an intensity modulated light-emitting diode and a lock-in amplifier-detector was used for the measurement of magnesium and phosphate in simulated plasma ultrafiltrate. Commercial clinical chemistry reagent kits were used as the analytic reagents. The sensitivity was demonstrated to be less than 0.2 pmole for magnesium and 1.2 pmole for phosphate.

The second colorimeter was designed for the specific mesurement of total calcium in solution. The optics were improved over the earlier instrument so that the system could be operated without the complexity of the lock-in amplifier. This made the instrument much more compact and less expensive for persons wishing to duplicate it. It performed very well for the measurement of total calcium in simulated kidney tubule fluid, rabbit sera, and human urines. Calcium content of the latter two sorts of samples were measured with commercial instruments (macro-scale) and the results agreed with the microcolormetric method within 5%. Careful attention to sample delivery has enabled the measurement of 5 picomoles of calcium in simulated tubule fluid with a precision of less than 2%. It has been found that measurement of the peak amplitude of the transmittance vs. time curve is as precise as the interal peak light measurement is easier than integration.

The third colorimeter was designed as a batch instrument for urea measurements. The working volume of the cuvet was about 800 nL; sample

size and concentration constraints were not as severe as with the earlier instruments. The batch approach was used because the reaction required exposure of sample and reagent to 100C for 10 minutes and because it was desirable to recover the sample, which could contain radio-labelled compounds, for other analyses. The instrument used an incandescent lamp and gelative filters, and a photodiode. A precision of better than 3% is achievable at the 50 pmole level.

Significance to Biomedical Research and the Program of the Institute:

We have demonstrated that picomole amounts of several materials of interest to biomedical research can be measured with good precision with this new flow-through colorimeter. Present techniques which may be tedious and require expensive and elaborate instrumentation now may be replaced by this simple colorimeter.

Proposed Course:

The development work has been completed and we are now encouraging commercial instrument manufacturers to produce a verison of the device.

Publications:

1. Vurek, G. G.: Flow-through nanocolorimeter for measurement of picomole amounts of magnesium and phosphate. Anal. Letters. 14(A4): 261-269, 1981.

2. Vurek, G. G.: Calcium measurement by continuous-flow colorimetry. Anal. Biochm. 114: (in press), 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01435-02 LTD

PERIOD COVERED October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

New Catheter Idea to Facilitate Radiologic Instrumentation

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

PI: R. L. Bowman Chief, LTD LTD NHLBI

COOPERATING UNITS (if any)
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The concept of topocatheter extension to the standard angiographic catheter to facilitate entry into small and tortuous vessels involves the production of a high strength thin walled tube that can be easily turned inside out. When the thin wall extension is exposed to negative pressure inside the catheter it first collapses and then inverts itself until it lines the distal end of the catheter. Pressure inside the catheter causes the lining to form a valve and shut so that flow can only occur by again inverting the thin wall tube. When the thin wall tube everts it rolls onto the inside of the vessel wall with no friction so that it can enter tortuous vessels with ease. A 12cm extension on a #7F catheter tapered to 1mm is typical. A triangular tapered shape reduces self interference of the thin wall extension.

PHS-6040
(Rev. 2-81)

Objectives:

To continue to develop ideas to improve the performance of angiographic catheters. Current activity involves the development of an extension to a conventional angiographic catheter to reach smaller vessels.

Methods:

A double wall "topocatheter" or rolling tube discribed last year and demonstrated to be workable only in #8F catheters with liberal lubrication. Efforts to provde various hydrophilic coatings prepared by BEIB did not perform adequately but while testing the double wall tube it was noted that another mode of extension and withdrawal could occur that might simplify the construction.

The double wall construction and its potential for guidance was put aside for later work and work was continued on the single wall system. Single wall topocatheter extension pieces were drawn from either Norton or Stevens polyurethane stock. Ether or ester stock of various formulations were drawn on a specially constructed apparatus to produce thin walled tapered tubes 3mm o.d. to 1 mm o.d. in about 10-12cm with wall thickness of about 25u.

The thin walled extensions attached to the end of the catheter would collapse and be drawn into the lumen of the catheter when suction was applied. Hydrostatic pressure collapses the extension and forces fluid into the catheter by rolling walls of the extension back inside the catheter until the catheter is lined with the thin wall piece and there is no longer a collapsed portion to prevent free flow into the catheter. The entry of the extension into the catheter involves no sliding friction as there is no relative wall motion.

In-flow is not impeded once the inflection is complete. On application of pressure inside the catheter the thin wall piece acts like a valve and causes hydrostatic pressure to build up between the catheter wall and the collapsed extenison. This allows the outflow from the catheter to unroll the thin wall extension against the blood vessel wall by pulling the collapsed portion out of the catheter and everting it against the vessel wall. This time there is no blood vessel wall extension friction as it simply everts into the lumen at the expense of length of collapsed extension. There is therefore no friction and there is complete freedom to negotiate great tortuosity. When fully extended the valving action is no longer active and there is no impediment to delivery of material from the catheter. The thin wall extension is distensable and may transmit enough pressure to widen narrow vessels to permit more rapid delivery of a "shot".

Removal of the extension can be reversed by application of suction or simply withdrawing it. There may be an advantage to suck the extension back within the catheter to avoid loss of adhesive into the larger vessels as the withdrawal is made.

The removal of an extended thin wall piece from a particularly tortuous vessel could be more difficult than the introduction as the removal is not done in the frictionless "rolling wall" mode that introduced it. The solution to this particular problem has already been anticipated and demonstrated by the double wall version alluded to at the beginning of this report. If the single wall extension can be made slippery enough there may not be any need for the more complex construction.

Major Findings:

Material problems have largely been solved by the use of polyurethane tube stock to draw and form the extension to strong thin extensions that have a special triangular cross section and are sufficiently tapered to reduce interference of outer and inner walls. Secure adhesion to introducing the catheter requires solvent welding to polyurethane catheters whereby the bond does not fail before the catheter. Polyether or polyester forms have both been tested and no significant hydrolyses has been noted in either in a matter of weeks of exposure to water and soaps. 15cm extensions tapering from #7 (2.5mm) to 1 mm with wall thickness of 25u are now easily drawn in a few minutes from inexpensive stock using a simple drawing machine easily constructed.

There may be some problem of lubrication for the use of this material as in the past it was not possible to get satisfactory easy reversable extension and withdrawal until the method of making a triangular configuration that avoids the problem of interference produced by a flat sheet entering a round hole. The new configuration may eliminate the need for lubrication as the system now functions without lubrication. If and when a suitable lubricant surface is applied there may be some advantages in getting out of very tortuous vessels.

Significance to Biomedical Research and the Program of the Institute:

Interventional radiographic angiography is now an established therapeutic procedure for a number of vascular and other diseases. Access to small and tortuous vessels is a currently is limited. Ease of entry may reduce the problems of direction by permitting repeated "stabs".

Proposed Course:

Practical testing of the catheter in cooperation with the Radiology Department and publication.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01437-02 LTD

PERIOD COVERED October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

New Continuous Extraction Method With A 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 Medical Officer LTD NHLBI

COOPERATING UNITS (if any)

None

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
A compact table top model of the coil planet centrifuge simultaneously enables both preliminary purification and enrichment of samples from crude extracts or biological fluids. The method uses hydrodynamic behavior of two immiscible solvent phases in a rotating coiled tube to retain the stationary phase against a high flow rate of the mobile phase. Consequently, a small quantity of the sample present in a large volume of the mobile phase is efficiently extracted into a small volume of the stationary phase within a short period of time and at a high recovery rate. The capability of the present method was demonstrated in the extraction of dinitrophenyl (DNP) amino acids (used as a comparative performance standard) with a set of two-phase solvent systems composed of ethyl acetate and 0.5M NaH_2PO_4.

PHS-6040
(Rev. 2-81)

Project Description: Development of continuous extraction scheme using the horizontal flow-through coil planet centrifuge.

Method Employed and Major Findings:

a) Principle

The present method takes advantage of the intriguing hydrodynamic behavior of two immiscible solvent phases in a coiled tube mounted around the rotary drum of the horizontal flow-through coil planet centrifuge. Centrifugation produces a hydrodynamic equilibrium of the two phases in the coil where one phase dominates at the head end of the coil and in many occasions two phases are completely separated along the length of the coil one phase entirely occupying the head side of the coil.

A series of observations has been made on various two-phase solvent systems having a wide spectrum of physical properties. The results so far indicate that the distribution of the two phases is affected by three major factors, i.e., wall affinity, relative density and viscosity of the two phases. The phase which has higher wall affinity, lower density and less viscosity tends to distribute itself toward the head of the coil. When all three requriements are satisfied, the upper phase will quickly move toward the head and the phase separation is completed in a short period of time. This ideal group of the solvent pair includes a number of useful extraction media such as hexane, ether, ethyl acetate, toluene, methylethyl ketone, benzene, etc., mixed with aqueous solution where various salts can be added to adjust the pH and ionic strength of the aqueous phase. Various third solvents such as methanol, acetic acid, etc., can also be added without altering the overall behavior of the two phases.

When the two phases (A & B) are completely separated in the coil with phase A on the head side and phase B on the tail side, continuous extraciton is possible in three ways. In the first method, the coil is filled with phase A (stationary phase) followed by elution with phase B (mobile phase containing sample) through the head end of the coil. Phase B then travels through phase A in the coil toward the tail. Consequently, the sample present in phase B is extracted into the stationary phase A and the stripped phase B is eluted through the tail of the coil. In the second operation, the coil is first filled with phase B (stationary phase) and phase A (mobile phase containing the sample) is pumped through the tail of the coil. Extraction process similarly takes place in the coil, the stripped phase A being eluted through the head of the coil. Third operation involves dual countercurrent extraction (not described here) in which phases A and B are simultaneously introduced into the coil through the tail and the head, respectively. In this case the coil should be equipped with an additional pair of flow tubes at each end to collect both enriched and stripped phases. If desirable, the sample solution may be fed into the coil through another flow line connected at the middle portion of the coil.

b) Apparatus

The design of the apparatus used in the present studies is similar to the toroidal coil planet centrifuge (Z0L HL 01437-01 LTD) which permits continuous elution without the use of rotating seals. The motor drives the rotary frame around the horizontal stationary pipe mounted on the axis of the centrifuge. The rotary frame consists of a pair of aluminum discs rigidly bridged together with multiple links and hold a pair of rotary column holders in the symmetrical positions 10 cm away from the central axis of the centrifuge. The shaft of each holder is equipped with a plastic planetary gear which is coupled to an identical sun gear mounted around the central stationary pipe. In order to provide mechanical stability, a short coupling pipe is coaxially mounted to the free end of the rotary frame while the other end of the coupling pipe is supported by a stationary wall member of the centrifuge through a ball bearing. The coiled column was made by winding the desired length of a PTFE tube around one of the holders while a counterweight is applied on the other holder to balance the centrifuge. A pair of flow tubes from the coiled column is first passed through the center hole of the holder shaft and led through the side hole of the short coupling pipe to reach the opening of the central stationary pipe. These flow tubes are thoroughly lubricated with silicone grease and protected with a piece of plastic tubing at each supported portion to prevent direct contact with metal parts. The revolutional speed can be regulated up to 1000 rpm. The apparatus is a compact table top model whose dimensions are approximately 16x16x17 inches.

c) Studies on Retention of the Stationary phase.

The capability of the present scheme in retaining a large amount of the stationary phase was demonstrated with a coiled column prepared from 2.5 m long, 2.6 mm i.d. PTFE tube which was coiled around a holder having a beta value of 0.75. The column consisted of 5 helical turns (6 inch helical diameter) and had a total capacity of about 15 ml. Typical two-phase solvent systems composed of ethyl acetate/H_2O and ethyl acetate/0.5M sodium phosphate (pH 4.4) at a volume ratio of 1:1 were selected. In each operation the coiled column was entirely filled with the stationary phase and the mobile phase was pumped at a rate of 516 ml/hr (maximum capacity of the pump) in the proper direction (head-tail elution for the aqueous phase and tail-head elution for nonaqueous phase) while the apparatus was run at a given revolutional speed. The retained stationary phase volume and its percentage relative to the total column capacity were calculated from the eluted stationary phase volume, total column capacity and the volume of the dead space in the flow path.

The overall results indicated that ideal retention levels of over 70% are provided at the revolutional speed of over 600 rpm for both phase systems with either the aqueous or nonaqueous phase used as the stationary phase. The results also suggest that much higher flow rate are applicable

with high revolutional speeds.

d) Continuous Extraction Experiments

A series of experiments has been performed to demonstrate the capability of the present scheme to extract a solute present in a large volume of the mobile phase into a small volume of the stationary phase retained in the coiled column. This requires a set of conditions such that the solute must favor partition to the stationary phase. With commonly used extraction media such as an ethyl acetate/aqueous system, partition coefficients of various biological materials can be conveniently adjusted by modifying the pH and/or ionic strength of the aqueous phase to meet the above requirement. For the present studies, a pair of DNP amino acids, N-DNP-L-leucine and delta-N-DNP-L-ornithine, were selected because they provided suitable partition coefficients for the present solvent systems composed of ethyl acetate/0.5M sodium phosphate. The experiments were performed with the coiled column used in the previous retention studies. The typical extraction procedure may be divided into three steps, i.e., extraction, cleaning-up, and collection. In each operation, the column was filled with the stationary phase and the mobile phase containing the sample was eluted through the column in the proper direction while the apparatus was run at 600 rpm. The extraction process was continued until 400 ml of the mobile phase was eluted. Then the mobile phase was replaced by the same phase but free of solute to wash the column contents. This cleaning process was continued until the additional 100 ml of the mobile phase was eluted. This would elute out all impurities having the partition coefficients of 0.1 or greater. The sample extracted into the stationary phase in the coiled column was conveniently collected by eluting with the mobile phase in the opposite direction. The degree of sample recovery was estimated by comparing the amount of the sample in the original mobile phase to that in the collected stationary phase.

The results of the experiments revealed that small amount of the sample ranging from 4mg% to 0.04mg% present in 400 ml of the mobile phase was efficiently extracted into about 10 ml of the stationary phase at a high recovery rate in less than one hour. As the sample concentration in the mobile phase was reduced from 4mg% to 0.04mg%, the sample recovery rate rose from 94% to nearly 100% indicating that no sample loss occurs due to the absorption effect.

Significance to Biomedical Research and the Program of the Institute

The overall experimental results indicate a potential usefulness of the present method in processing a large amount of crude extracts or biological fluids in research laboratories. A small amount of the sample present in several hundred milliliters of the original solution can be enriched in 10 ml of the nonaqueous phase free of salt in one hour at a high recovery rate. The method would be easily scaled up to deal with liters of solvents. Typical examples of biomedical applications of this

method may be the separation of drugs and their metabolites from urine samples, purification of antibiotics and other compounds of interest from culture medium, purification of minute amounts of biologically active principles from curede extracts of plant or animal tissues.

Proposed Course

1. Application of the method to biomedical research.

2. Refinement of the apparatus.

Publications:

1. Ito, Y.: New Continuous Extraction Method with a Coil Planet Centrifuge. J. Chromatogr. 207: 161-169, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01438-02 LTD

PERIOD COVERED October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Preparative Countercurrent Chromatography With A Rotating Coil Assembly

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

PI:	Y. Ito	Medical Officer	LTD	NHLBI
Other:	P. Carmeci	Electric Engineer	LTD	NHLBI
	Rohit Bhatnagar	Biological Aid	LTD	NHLBI

COOPERATING UNITS (if any)

None

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NHLBI, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

We have designed a simple bench top model of a countercurrent chromatograph which performs efficient preparative separations without the use of solid supports. The stationary phase is retained by gravity in a large diameter coil which rotates to promote efficient mixing of the two phases. Continuous elution of the mobile phase is accomplished without the use of rotating seals. We demonstrated the efficiency of the system by separating gram quantities of DNP (dinitrophenyl) amino acids. The design and construction of the apparatus should permit easy increases in scale for industrial applications.

PHS-6040
(Rev. 2-81)

Methods Employed and Major Findings

a) Principle

The present scheme uses a coiled tube which slowly rotates around its horizontal axis with respect to the gravitational field. Particles introduced in such a coil move toward one end of the coil. This end is defined as the head and the other end, the tail of the coil. A two-phase solvent system confined in this rotating coil distributes itself in such a way that nearly equal volumes of the two phases occupy each helical turn while any excess of either phase remains at the tail. This hydrodynamic behavior of the solvents allows elution of either phase through the head while retaining a large amount of the stationary phase in each turn of the coil. Consequently, solutes introduced through the head of the coil are subjected to an efficient partition process between the mobile and stationary phases in each turn of the coil and are eluted through the tail in the order of their partition coefficients as in liquid chromatography but in the absence of solid supports.

b) Apparatus

The motor drives a rotary frame through a pair of toothed pulleys coupled with a toothed belt. The rotary frame consists of three aluminum arms rigidly bridged together with links and holds two rotary elements, the countershaft and the centrally located column holder assembly. The countershaft is equipped with a toothed pulley at one end and a plastic gear at the other end. The pulley of the countershaft is coupled with a toothed belt to an identical stationary pulley mounted onto the stationary wall member of the central axis of the apparatus. This coupling causes a counter-rotation of the countershaft on the rotary frame. This motion is further conveyed to the central column holder assembly by 1:1 gear coupling. Consequently, the column holder assembly rotates around its own axis at a rate twice that of the rotary frame in the same direction. This particular design gives a great advantage in that the scheme allows the flow in and out of the rotating column without the use of rotating seals.

Separation columns used in the present studies consist of coiled glass tubes of 0.5 cm i.d. with different helical diameters. One column has a 2.5 cm helical diameter with a 90 ml capacity and the other has a 1.25 cm helical diameter with a 45 ml capacity. Both columns contain approximately 50 helical turns. Each column is supported by a hollow aluminum core of the suitable diameter which is in turn mounted onto the column holder by a screw at each end. The column holder is equipped with two different levels for mounting columns, the first level being located 6.5 cm from the central axis of the apparatus and the second level, 13 cm from the same axis. A maximum of 30 columns at the second level. The desired number of columns can be connected in series with a short piece of heat shirnkable PTFE tubing at each junction.

Flow tubes from the column are first led through the center hole of

of the column holder shaft, then passed through a pair of holes at the periphery of the rotary arms, and finally supported by a stationary tube support located at the central axis of the apparatus. These tubes are thoroughly lubricated with silicone grease and protected with a piece of plastic tubing to prevent contact with metal parts.

The rotational speed of the column assembly can be regulated up to 300 rpm. However, in the present studies, fragility of the glass column limits the maximum rate down to approximately 100 rpm. A Beckman Accu Pump and Chromatronix Metering Pump are used to elute the solvents and an LKB Uvicord III to monitor the eluate at 280 nm.

c) Preliminary Studies on Partition Capability

The performance of the present countercurrent chromatographic scheme was investigated by measuring the degree of stationary phase retention and partition efficiency. The two types of coils with 1.25 cm and 2.5 cm o.d. cores were tested, and each mounted in both inner and outer positions of the column holder.

The degree of retention of the stationary phase in each column was measured with a two-phase solvent system composed of $CHCl_3/CH_3COOH/H_2O$ at a 2:2:1 volume ratio under various rotational speeds and flow rates. The results clearly show that the retention levels of the nonaqueous phase are substantially lower than those of the aqueous phase throughout the applied rotational speeds. This higher level of retention produced by the aqueous phase may be largely attributed to this greater affinity to the glass wall of the column. The effects of the flow rate on the retention of the stationary phase are also clearly demonstrated; the slower the flow, the higher the retention levels. The retention levels produced by the small coil is substantially higher than those by the large coil for both nonaqueous and aqueous stationary phases. This may indicate that in the small core coil the linear velocity relative to the gravity becomes smaller resulting in less violent mixing of the two phases and, therefore, higher levels of phase retention at a given rotational speed occur. Data obtained with the same columns mounted in the inner and outer positions of the column holder gave similar results. Overall results indicate that satisfactory retention levels can be obtained with either type of coils under a wide range of rotational speeds and flow rates.

The partition efficiency of each column was evaluated with a two-phase solvent system composed of $CHCl_3/CH_3COOH/0.1N\ HCl$ at a 2:2:1 volume ratio and a pair of DNP amino acids as test samples. The two-phase solvent system was equilibrated in a separatory funnel at room temperature and separated before use. The sample solution was prepared by dissolving N-DNP-DL-glutamic acid and N-2,4-DNP-L-alanine in the upper aqueous phase to obtain the 0.5g% concentration of each component. In each separation the column was first filled with the stationary phase. This was followed by injection of 0.5 ml of the sample solution through the sample port

located on the flow line between the pump and the inlet of the column. Then the mobile phase was pumped through the head of the column while the column was rotated at a given rate. The eluate through the outlet of the column was continuously monitored with an LKB Uvicord III at 280 nm. Separations were performed under a wide range of operational conditions, of rotational speeds (0-80 rpm) and flow rates (120-240 ml/hr), while both upper aqueous and lower nonaqueous phases were tested as the stationary phase.

The results show that the partition efficiency sharply increases as the rotational speed increases from 0 to 40-60 rpm, where the peak resolution becomes maximum. Further increase of the rotational speed to 80 rpm results in the loss of peak resolution. The optimum rotational speed thus ranges from 40 to 60 rpm for all groups. The highest peak resolution is given by the large coil while the small coil could yield much higher resolution if two coils are connected to make the capacity equal to that of the single large coil. The results obtained with a higher flow rate of 240 ml/hr yielded less efficient separations in both small and large coils compared with those produced at 120 ml/hr. The results obtained from the coils mounted in the inner position of the holder gave separations similar to those produced by respective coils mounted in the outer position of the column holder.

d) Preparative Countercurrent Chromatography with a Long Column

The preparative capability of the present scheme was examined with a long column consisting of 10 coils with 2.5 cm core o.d. connected in series (tail-head connection). The column consisted of nearly 500 helical turns with a total capacity of approximately 900 ml. It was symmetrically mounted on the outer positions of the column holder. The solvent system and the samples were the same as those used in the partition efficiency studies. The sample solution was prepared by dissolving 500 mg of each DNP amino acid for a total of 1 gram in 30 ml of the solvent consisting of equal amounts of the upper and lower phases. In each separation, the column was first filled with the stationary phase followed by sample injection through the sample port. Then the mobile phase was pumped though the head of the column while the column was rotated at the optimum rate determined by the preliminary studies. The separations were performed at a 120 ml/hr flow rate using both nonaqueous and aqueous stationary phases. In a chromatogram obtained at the optimum rotational speed of 40 rpm by using the nonaqueous stationary phase, the two DNP amino acids were completely resolved as symmetrical peaks and eluted out within 9 hours. The partition efficiency measures 1250 T.P. (theoretical plates) for the first peak and T.P. for the second peak. In a similar chromatogram obtained at 60 rpm using the aqueous phase as the stationary phase, the peak resolution is much greater than that of the separation using the nonaqueous stationary phase probably due to a much higher level of retention. The partition efficiency in the latter separation gives lower figures of 1000 T.P. for the first peak and 830 T.P. for the second peak.

Significance to Biomedical Research and the Program of the Institute

The present scheme enables preparative-scale separations with a simple, compact apparatus. Separations are performed without the presence of solid supports and, therefore, complications such as sample loss, contamination, and tailing of the solute peaks are minimized. The scheme yields high partition efficiency comparable to liquid chromatography while retaining high reproducibility and predictability inherent in the Craig countercurrent distribution method. Therefore, the present method would be very useful in separation and purification of biological materials.

Proposed Course

1. Application of the method to separation and purification of biological materials.

2. Refinement and improvement of the present scheme.

Publications

1. Ito, Y., and Bhatnagar, R.: Preparative Counter-current Chromatography With A Rotating Coil Assembly: J. Chromatogr., 207:171-180, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01439-01 LTD

PERIOD COVERED October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Biomedical and Biochemical Applications of Countercurrent Chromatography

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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NHLBI, NIH, Bethesda, Maryland 20205

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Various countercurrent chromatographic methods have been successfully applied to separation and purification of various biological materials such as peptide, daunorubicin derivatives, prostaglandins, various plant hormones, E. coli DNA, etc. The overall results indicated that the methods are extremely versatile and would become essential when other separation methods fail to provide satisfactory results.

PHS-6040
(Rev. 2-81)

Project Description:

Separation and purificaiton of biological samples of interest with various types of countercurrent chromatographs developed in our laboratory.

Methods and Major Findings:

Separation and purification of samples were carried out with various types of countercurrent chromatographs which include the new horizontal flow-through coil planet centrifuge (Z01 HL 01428-01 LTD), the toroidal coil planet centrifuge (Z01 HL 01437-01 LTD) and the non-synchronous flow-through coil planet centrifuge without rotating seals (Z01 HL 01436-01 LTD), all developed in our laboratory. These countercurrent chromatographs have a common feature in that the scheme uses a coiled separation column which rotates in a centrifugal acceleration field. The rotation of the coiled column facilitates both retention of the stationary phase and mixing of the two phases in each coil unit while the mobile phase is continuously eluted through the column. Consequently, solutes introduced in the inlet of the column are subjected to an efficient partition process between the mobile and the stationary phases in each coil unit and finally separated out according to their relative partition coefficients. In each separation, the column is first filled with the stationary phase (either upper or lower phase) followed by sample injection through the sample port. Then the column is rotated at a constant speed while the mobile phase is introduced into the column at the optimum flow rate. The eluate through the outlet of the column is continuously monitored with an U.V. monitor and/or fractionated with a fraction collector for further analysis. Biological samples applied in the present studies cover a broad spectrum ranging from small molecules to macromolecules. Some samples have produced problems such as sample loss and contamination when applied to high pressure liquid chromatography.

a) Peptide:

A synthesized peptide des enkephalin gamma-endorphin (Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu) was applied to countercurrent chromatography with a two-phase solvent system composed of n-BuOH/0.1% trifluoroacetic acid at a 1:1 volume ratio (K=0.19). The new horizontal flow-through coil planet centrifuge with a preparative column on the gear-side (1000 coil units and 260 ml total capacity) was employed. The apparatus was rotated at 400 rpm while the upper mobile phase was pumped into the column at 24 ml/hr. Although the peak resolution was not complete, the major peak was found to be highly purified. The sample recovery rate was 65% compared with 29% obtained by HPLC.

b) Daunorubicin Derivatives:

Countercurrent chromatographic separation of daunorubicin reduction

products was performed with the new horizontal flow-thrugh coil planet centrifuge equipped with a preparative column on the gear-side column holder. A two-phase solvent system composed of chloroform/ethylene chloride/hexane/methanol/water (1:1:1:3.5=1) was selected because it provides high solubility and suitable partition coefficients for the aglycones. The sample solution was prepared by dissoving the dried reduction products in 5 ml of the above solvent system consisting of equal amounts of the aqueous and nonaqueous phases. The separation was carried out at 400 rpm by pumping the upper aqueous phase at 24 ml/hr. The chromatogram obtained showed completely resolved three major peaks. Subsequent mass pectrometric analysis of these fractions could successfully identify these components as daunorubicin (D_1), 7-deoxydaunorubicin aglycone (dD_4), and 7-deoxydaunorubicinol aglycone (dD_3). Identification of these products isolated by HPLC was not successful due to contamination of solid support materials into the fractions.

c) Prostaglandin Metabolites:

Various forms of prostaglandins have been separated by countercurrent chromatography with a two-phase solvent system composed of chloroform/acetic acid/water at a 2:2:1 volume ratio. The new horizontal flow-through coil planet centrifuge equipped with an analytical column on the pulley-side holder was used for separation. Highly efficient separations obtained from these experiments suggest that the method could be applied for the metabolic studies of prostaglandins since the precursor compounds, arachidonic acid and eicosatrienoic acid, are completely separated from the prostaglandins formed from them. The sample used in the present study was the supernatant solution from the incubation of human platelets with arachidonic acid labelled with carbon 14. Formic acid acidified supernatant fluid was extracted three times with 2 volumes of ethyl acetate after the addition of carrier prostaglandin. The combined extracts were used after blowing to dryness with nitrogen and dissovling in the upper phase of the above two phase solvent system. The separation was performed at 450 rpm using the upper aqueous phase as the mobile phase. The metabolite, TXB_2, was eluted out with a sharp peak at close to the solvent front while the precursor, arachidonic acid, were long retained in the column and finally recovered from the column after stopping the centifugation. TXB_2 consisted of only 1% of the original compound and yet complete reproducible recovery was achieved.

d) Plant Hormones:

Countercurrent chromatography was applied to separations of various plant hormones such as gibberellins, auxins, cytokinins and abscisic acid. A toroidal coil planet centrifuge with PTFE tubing (8,500 turns, 0.55 mm i.d., helical diamter 1.5 mm, total solvent capacity 18 ml, β=0.75) was used. The instrument was operated at 450-500 rpm (theoretical plates: 2000-6000) and the mobile phase was pumped at 2.4 ml/hr. Separation of compounds was monitored either at 256 or 210 nm with an UV monitor.

Based on the solvent systems employed, plant hormones and their metabolites and conjugates exhibit remarkable differences in partition coefficients (P.C.) and these serve as a useful guide for their successful separation by countercurrent chromatography. For example, several indoles of auxin type have partition coefficients (upper phase/lower phase) ranging from 3.83 for indole-3-acetamide to 0.33 for indole-3-acetonitrile in a solvent system containing hexane-ethyl acetate-methanol-water (0.6:1.4:1.0:1.0). In this system, other indoles such as indole-3-acetic acid (P.C. 1.01), indole-3-propionic acid (0.50), indole-3-butyric acid (0.57), indole-3-acrylic acid (1.14) and indole-3-carboxylic acid (1.61) have different partition coefficients. When a mixture containing these indoles were chromatograhed using organic (upper) phase as stationary, they were completely separated. Further, their retention times (r.t.) could be correlated to the P.C. (the higher the P.C. the shorter the r.t. in this solvent mode). These indoles could also be separated in a solvent system containing chloroform-acetic acid-water (2:2:1). The growth inhibitor, abscisic acid (ABA) (P.C. 0.56), was separated from other indoles including the most commonly occurring auxin, indole-3-acetic acid (P.C. 0.94), in this solvent system while keeping organic phase as stationary.

Gibberellins presented a few problems because of limited choice of solvent systems and also because of lack of chromophores for UV detection. However, they (GA_3, GA_4, and GA_7) were successfully separated in a solvent system containing ether-methanol-phosphate buffer, pH 7 (3:1:2) and the separated compounds were monitored by UV absorption at 210 nm and by bioassay. This method was found to be very suitable for the separation of cytokinins which include 6-(ν,ν-dimethylallylamino) purine (DMAA) and Zeatin (Z), and their corresponding ribosides whose partition coefficients (up/lp) in ethyl acetate-methanol-0.05M phosphate buffer, pH 7 (3:1:3) range from a 0.2 for DMAA to 4.25 for Zeatin riboside (ZR). Zeatin and DMAA riboside (DMAAR) have P.C.s of 2.26 and 0.73, respectively. When these compounds were chromatographed keeping non-aqueous phase as stationary, as expected ZR being the highest P.C., eluted first and DMAA eluted last. The other two compounds, namely ZR and DMAA appeared between ZR and DMAA. When the phase was reversed, i.e., keeping aqueous phase as stationary, the separation profile also reversed as expected, since the P.C.s in this case were the inverse of those reported values. In other words, in the chromatographic profile, DMAA and its riboside eluted first and then came Zeatin and its riboside. The utilization of countercurrent chromatography was successful for analysis of ABA and other compounds from plant tissue, and this method was found either comparable to (e.g. ABA) or superior to (e.g. gibberellins) HPLC.

e) DNA (E. coli):

Separation of E. coli DNA from RNA and other compounds was performed with an Albertsson's polymer phase system composed of 5% dextran T 500, 4% polyethylene glycol 6000, and 10 mM sodium phosphate. The non-synchronous flow-through coil planet centrifuge without rotating seals was used for

separation. The apparatus has been improved by a new design which gives much less vibration and noise even at a higher revolutional speed of 1000 rpm. The coiled column consisted of 600 helical turns (0.67 cm helical diameter) of 1 mm i.d. PTFE tubing with a total capacity of about 15 ml. Separation was performed at 1000 rpm revolution and 5 rpm coil rotation. The upper phase was pumped at 8.5 ml/hr flow rate. DNA with a high partition coefficient was eluted close to the solvent front with a sharp peak and almost completely separated from RNA and other compounds which were eluted later with much broader peaks.

Significance to Biomedical Research and the Program of the Institute:

Separation and purification of biological samples are essential for most of the biomedical studies. We have deomonstrated versatility, high efficiency, excellent recovery and reproducibility of countercurrent chromatography using various biologically important samples. Countercurrent chromatography offers an ideal alternative separation method when other methods fail or produce problems.

Proposed Course:

Further application of the method to various biological samples.

Publications:

1. Nakazawa, H., Andrews, P. A., Bachur, N. R., and Ito, Y.: Isolation of Daunorubicin Derivatives by Counter-current Chromatography with the Horizontal Flow-through Coil Planet Centrifuge. J. Chromatogr. 205 (1981) 482-485.

2. Knight, M., Ito, Y., and Chase, T. N.: Preparative Purification of the Peptide des Enkephalin Gamma-endorphin: Comparison of High Performance Liquid Chromatography and Countercurrent Chromatography. J. Chromatogr., in press.

3. Brown, E. A. B. and Ito, Y.: Separation of Prostaglandins Using the New Horizontal Flow-through Coil Planet Centrifuge. J. Biochem. Biophys. Methods 3 (1980) 77-87.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01440-01 LTD

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Microfluorometer for Analysis of Femtomole Amounts of Biochemicals

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

PI: G.G. Vurek	Sr. Investigator	LTD NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Technical Development

SECTION

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NIH, NHLBI, Bethesda, MD 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A new flow-through micro-fluorometer with a working volume of less than 250 nL has been developed. The cuvette is closely coupled to the photomultiplier detector to insure efficient collection of the fluorescence. Excitation energy is brought to the cuvet via a low-loss optical fiber; the light source is a 100 watt mercury arc. Tests have demonstrated the fluorometer's ability to measure concentrations of fluorescein below 10^{-9} M/L and total amounts of less than 10^{-14} moles injected into a flowing stream of buffer. When used as part of a continuous flow analysis instrument the fluorometer should be valuable for femtomole level analysis of amino acids and enzyme substrates.

PHS-6040
(Rev. 2-81)

Methods Employed:

The cuvet is formed of Suprasil quartz, which has low intrinsic fluorescence. In contrast to other workers' designs, the new microfluorometer uses a fiber optic to bring excitation energy to sample and places the cuvet close to the photomultiplier for efficient collection of the fluorescence. The cuvet is part of a continuous flow analysis system. A syringe-type withdrawal pump pulls reagent from a reservoir past an injection port to the pump. Samples are injected into the reagent stream. For analysis of samples which have intrinsic fluorescence, a fast flow rate can be used; a slow flow rate can be used where the fluorescent product requires time to develop, as in enzyme assays.

Major Findings:

Tests have been made to establish the sensitivity of the fluorometer. Both fluorescein and NADH have been used. The former has a high quantum efficiency and the latter is a biologically important cofactor. Using a simple commercial photometer as the detector, fluorescein at concentrations below 10^{-9} M/L could be distinguished from the background and NADH was detectable at the 10^{-7} M/L level. Because the cuvet volume is about 3×10^{-7} L, the absolute sensitivity is less than 3×10^{-16} moles for fluorescein and 3×10^{-14} moles for NADH.

To test the systems potential for enzyme analysis, in which some time may be needed for the reaction to generate significant changes in fluorescence, a pumping speed of 0.9 uL/min. was used. Injections of approximately 10 nL of 6 uM/L fluorescein demonstrated delays of 15 minutes were achievable and the width of the peak, at the 1% of peak height, was about four minutes.

Significance to Biomedical Research and the Program of the Institute:

This new microfluorometer will permit easy measurement of compounds at sub-picomole levels. This means that the content of single cells or very small portions of tissues can be analyzed without expensive instruments or time-consuming enzyme multiplication procedures. Biochemistry at the cellular level should be greatly facilitated.

Proposed Course:

Quantitative excitation of the apparatus will proceed and its performance for assay of several materials including amino acids and ammonium ions will be demonstrated.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01441-01 LTD

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Measurement of Picomole Amounts of Ammonium Ion

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

PI: Gerald G. Vurek   Sr. Investigator   LTD NHLBI

COOPERATING UNITS (if any)
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LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, MD 20205

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1	1	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Techniques for measurement of picomole amounts of ammonium ion or ammonia are being explored. One highly sensitive approach involves the use of sensitized mercury luminescence. This approach has good sensitivity but quenching of the luminescence gives problems with reproducibility. Both oxygen and water vapor quench significantly and must be excluded. An alternative technique, an enzymatic method involving the conversion of ammonium ion and α-ketoglutarate to glutamic acid coupled to the fluorometric detection of changes in NADH offers good sensitivity and specificity. This technique requires the use of a new micro-fluorometer, described elsewhere.

PHS-6040
(Rev. 2-81)

Methods Employed:

Mercury vapor in nitrogen gas can be raised to an excited state by illuminating the mixture with light from a low-pressure mercury lamp. The excited state mercury can react with ammonia gas to form an excited complex, which decomposes with the release of a photon at 345 nm and neutral mercury and ammonia. The method is highly sensitive, but is best fitted measurement of ammonia in dry, oxygen free gas. Ammonim ions in biologic fluids can be converted to ammonia gas by injecting the samples into strong alkali. Another method involves the enzymatic conversion of ammonia and α-keto-glutarate to glutamic acid. The reaction is accompanied by a stoichiometric conversion of NADH to NAD, which can be measured fluorometrically in a new microfluorometer.

Major Findings:

Preliminary test showed that picomole amounts of ammonium ions in solution could be converted to ammonia gas and measured by the sensitized mercury technique. However, two major difficulties are currently unresolved. One is the slow release of ammonia from the injected solution. This process requires more than 15 min to complete. The second problem relates to the quenching of the luminescence by oxygen and water vapor. Both these gases can divert energy from excited state mercury atoms and the mercury-ammonia complex and prevent emission of the luminescence. Effort is being directed toward the elimination of these two problems.

As an alternate approach, an enzymatic procedure is being developed. Ammonium ions can be reacted with a-ketoglutarate to form glutamic acid in the presence of glutamic dehydrogenase. In the process NADH is oxidized to NAD and the change in the fluorescence of the former is measurable. A new microfluorometer is described elsewhere in detail. In summary, it has a working volume of about 300 nL and can measure NADH at concentrations down to 10^{-7} M/L. The fluorometer can be part of a continuous flow analysis system arranged so that the delay between injection and measurement can easily be long enough for the reaction to occur. Samples can be injected at suitable intervals so that reactions can be occuring along the pipeline during the delay intervals.

Significance to Biomedical Research and the Program of the Institute:

The urinary excretion of ammonia is related to a variety of factors and the development of a method for picomole amounts of ammonia would facilitate the study of renal handling of that material at the level of isolated tubules.

Proposed Course:

Effort will be directed to improve the response time and reduce the quenching effects of oxygen and water vapor. Also, the enzymatic method will be pursued and evaluated.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01442-01 LTD

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)
Urinary Valve Prosthesis

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

PI:	R.L. Bowman	Chief, LTD	LTD NHLBI
	A.L. Swain	Biological Aid	LTD NHLBI

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A urinary prosthetic valve for intraurethral installation in the neck of the bladder via the urethra was constructed of silicon elastomers by simple "hand lay up" procedures. The valve action is provided by a double wall that is forced to shut off the channel by pressure transmitted from the distended cuff that provides a retention enlargement in the neck of the bladder. Pressure applied to the closed valve displaces the fluid from the valve into the higher pressure retention reservoir thru a check valve in a communicating channel. When finger pressure is removed from the "sphincter" urine flows unimpeded. Flow from the high pressure cuff back into the sphincter slowly refills the sphincter thru a restriction to provide action to empty the bladder. The action can be repeated if necessary immediately.

PHS-6040
(Rev. 2-81)

Objectives:

To provide a prosthetic urinary valve that will reduce the threat of ascending urinary infection and eliminate the external tube and clamp. It is assumed that catheter facilitates the ascent of infection and is an undesirable nuisance.

Project Description:

The prosthesis consists of a semi-rigid silicone tube (approx. 5mm in diameter, 50mm in length) with an inflatable cuff at the top end which holds about 13cc fluid. At the bottom end there is a smaller inflatable chamber with thin inner walls that, when filled with fluid (approx. 1cc), will close in on the tubular passage, thus blocking it off. These two chambers are connected by a one-way "leaky" valve that opens up in, and allows easy flow of fluid into the top chamber. When the fluid-filled lower chamber is squeezed, the contents is forced up into the top chamber. Because of the higher pressure in the top chamber, the fluid can drain slowly back down to fill the bottom chamber through the leaky valve, thereby blocking off the passage of the urine.

The upper chamber will rest in the bladder neck with the tube extending down the urethral lumen as far as the membranous area. By pressing at that specific point on the skin, the lower chamber is squeezed and the fluid from it is forced through the check valve into the higher pressure upper chamber and remains there (as urine flows down the open passage) until the fluid slowly drains back down through the leak and thus reinflates the lower chamber to block off the outflow of urine. The leak can be adjusted to provide time to drain the bladder and the process can be repeated immediately, if necessary.

Methods Employed:

After experimenting with many different materials, Silicone 9590 was decidedly the best material for this prosthesis. The whole device is made of Silicone 9590, a heat-curing rubber with a catalyst in a 1:1 ratio. The silicone is spread over a mandrel and cured at 130°C for at least 10 minutes. After cutting the cured form off the mandrel and rearranging it in the proper form, uncured Silicone 9590 is used as the cement and the whole device is again cured by heat.

The "leaky" valve is made by coating a piece of wire that has been flattened at one end with Silicone 9590, curing it, and removing it from the wire. Holes are punched in the valve neck, or a corner of the mouth of the valve is cut off to cause the slow leak. Other procedures for causing the return flow were employed, such as inserting a high-resistance connecting rube between the chambers. Because of the possibility of that tube getting obstructed, the "leaky" valve seems to be the best approach.

Major Findings:

By proper selection of the method to be used for the leak, the delay time for the valve of the lower chamber to close after being squeezed can be adjusted to approximately 90 seconds.

Significance to Biomedical Research and the Program of the Institute:

This prosthesis can be an effective alternative to prostheses involving surgical procedures or the use of retention catheters in males where the channels between the catheter and the urethra invite ascending urinary infection. It could be custom-made for each individual and the cost and time required for this procedure will be minimal.

Proposed Course:

Publication and future development in cooperation with a urological service.

Publications:

None

Annual Report of the Pathology Branch
Division of Intramural Research
National Heart, Lung, and Blood Institute
October 1, 1980 through September 30, 1981

Investigations during the above time period as in previous years centered primarily on studies of coronary, valvular, congenital, and myocardial heart diseases.

CORONARY HEART DISEASE

Last year we compared the amount and extent of coronary narrowing by atherosclerotic plaque at necropsy in 22 patients with acute anterior wall myocardial infarction to 28 patients with posterior wall acute myocardial infarction. This year we extended that study by comparing the amount and extent of coronary narrowing by atherosclerotic plaque and of myocardial scarring at necropsy in 22 patients with anterior wall healed myocardial infarction to 37 patients with healed posterior wall myocardial infarction. As in previous studies in recent years from this unit, the 4 major coronary arteries were examined by cutting them into 5-mm segments and preparing a histologic section from each one. The mean percent of 5-mm segments narrowed severely from all 4 major arteries was similar in the anterior and posterior myocardial infarction groups (38%-vs-46%). The patients with anterior myocardial infarction, however, had a higher percentage of 5-mm segments of the left anterior descending coronary arteries severely narrowed than the left circumflex but not the right coronary artery. The patients with posterior myocardial infarction has a higher percentage of segments of the right and left circumflex coronary arteries severely narrowed then the left anterior descending. The anterior myocardial infarction group had larger left ventricular scars than the posterior myocardial infarction group.

Cardiac arrest in the cardiac catheterization laboratory in patients with angina pectoris is, of course, unusual. During the past 10 years we examined at necropsy 10 patients with angina pectoris who had fatal cardiac arrest during cardiac catheterization. Nine died during attempted coronary angiography and the remaining patient during right sided catheterization. All 10 patients had at least 3 of the 4 major epicardial coronary arteries narrowed 76-100% in cross-sectional area by atherosclerotic plaques, including 7 with this degree of narrowing of the left main coronary artery by plaque: 2 additional patients had severe narrowing of the left main coronary artery by thromboemboli material superimposed on small plaques. Division of the four major coronary arteries into 5-mm segments disclosed that 9% of the segments were narrowed 96-100%; 49%, 76-95%; 23%, 51-75%; 13%, 26-50%, and only 6 segments 25% or less. Thus, patients with angina

pectoris who die during cardiac catheterization have particularly severe and diffuse coronary atherosclerotosis and usually severe narrowing of the left main coronary artery.

Last year we analyzed the amount of coronary narrowing quantitatively in a group of patients with type II and in another group with type IV hyperlipoproteinemia. This year we determined the amount of narrowing in each 5-mm segment of the 4 major coronary arteries in 5 patients with type III hyperlipoproteinemia, all of whom had fatal coronary heart disease. Of the 4 major coronary arteries, all 4 were narrowed 76-100% in cross-sectional area by plaques in 2 patients, 3 were narrowed to this degree in 2 patients and 2 were so narrowed in 1 patient. The mean percent of 5-mm long segments of coronary artery narrowed: 96-100% was 14; 76-95%, mean 35; 51-75%, 24; 26-50%, 16, and 0-25%, 11. Utilizing a scoring system of 1 to 4 for the 4 categories of narrowing (1=0-25%, 2=26-50%, 3=51-75% and 4=76-100% cross-sectional area narrowing), the scores per 5-mm segment for each patient ranged from 2.5-3.9 (mean 3.1).

Relatively little information is available on long term follow-up on patients who have had acute myocardial infarction many years earlier. We studied 8 patients who lived 20 to 31 years (mean 24) after healing of a transmural acute myocardial infarction. Two had left ventricular aneurysms and one had both right and left ventricular infarcts. A review of previous reports disclosed that survival for 2 decades or longer after healing of transmural acute myocardial infarction has rarely been documented and descriptions of hearts at necropsy in patients with well documented infarcts 20 years or more earlier are non-existent prior to our report.

Most patients now undergoing aortocoronary bypass operations receive at least 2 conduits between aorta and coronary artery. In the earlier days of aortocoronary bypass operations, however, it was common to insert only one conduit. The proper number of aortocoronary conduits to insert in patients undergoing this operative procedure has never been completely clarified. To obtain some information related to this subject we studied at necropsy 32 patients who died within 30 days of an aortocoronary bypass operation performed for angina pectoris. We compared the amount of narrowing in the nonbypassed to bypassed coronary arteries. The lumens in 42 (95%) of 44 nonbypassed and in 52 (100%) of 52 bypassed arteries were narrowed 76-100% in cross-sectional by atherosclerotic plaque. Of 616 five mm segments of the 44 nonbypassed arteries examined histologically, 292 (47%) were narrowed 76 to 100% in cross-sectional area by atherosclerotic plaque; of 728 segments examined in the 52 bypassed arteries, 375 (52%) wer similarly narrowed. Thirty-two (73%) of the 44 nonbypassed coronary arteries (in 23 patients) had been judged to be narrowed 50% or less in diameter on preoperative coronary angiography, but at necropsy 31 (97%) of these arteries were narrowed 76 to 100% in cross-sectional area and the other artery was narrowed 51 to 75%. Thus, significant amounts of

atherosclerotic plaque tend to be present at necropsy in all 3 major coronary systems of patients with angina pectoris who die early after an aortocoronary bypass operation.

VALVULAR HEART DISEASE

In recent years several echocardiographic papers have appeared describing echocardiographic features in patients with active infective endocarditis. Previous reports always lumped together patients with active infection on a heart valve irrespective of the valve infected and as a consequence a variable findings were reported. We described echocardiographic features in 27 patients with active infective endocarditis of the mitral valve followed up to 144 months after healing by M-mode echocardiograms.' The following findings were observed: 1. Little to no change occurred in the echocardiographic size of the vegetations during the first 6 weeks after diagnosis and institution of appropriate antibiotic therapy unless a major systemic embolus occurred. 2) The echocardiographic size of the vegetations did not determine the amount of cardiac damage or dysfunction produced by the valvular infection. 3) The larger the vegetations by echocardiogram, the greater was the likelihood of a clinical event compatible with a systemic embolus. 4) The gravest prognostic sign yielded by the echocardiogram was evidence of rupture of chordae tendineae. 5) Although a useful adjunct to diagnosis before appropriate antibiotic therapy was instituted, once bacteriologic cure was achieved, the echocardiogram was of limited value in delineating an active from a healed vegetation. 6) The echocardiographic appearance of the vegetation was not determined by the type of infecting bacterium.

We also examined 37 M-mode echocardiograms recorded during the period of active bacterial endocarditis involving the aortic valve in 17 patients. One or more echocardiographic abnormalities involving the aortic valve cusps or their immediate vicinity was observed in 15 (88%), including echos indicative of vegetations in 12 (71%). Of the 12 patients with echocardiographic evidence of aortic valve vegetation, 11 developed overt congestive heart failure and either died or had aortic valve replcement and 7 had clinical events compatible with systemic emboli; of the 5 patients without echo-demonstrated vegetatiors, only 1 had congestive heart failure, none had aortic valve replacement, 2 died, and 1 had a systemic embolus. In comparison to our previously reported echocardiographic observations in patients with active infective endocarditis involving either the mitral (29 patients) or tricuspid valve (23 patients), infection involving the aortic valve was far more liable to produce overt congestive heart failure and systemi emboli, to necessitate valve replacement, and to cause death during the period of active infection.

Mitral anular calcification is common in elderly people residing in high lipid environs. Usually by chest radiograph the anular deposits take the form of the J or U configuration. We studied two patients in whom anular calcium completely circled the mitral anulus

forming a complete O by extending across the anterior mitral leaflet. Such extension had not been described previously.

It is well known that many patients with systemic hypertension have a minimal or mild aortic regurgitation. Reports describing severe aortic regurgitation secondary to systemic hypertension alone have not been described. During the past 2 years, however, we observed 4 patients in whom severe aortic reguirigtation appeared to be the sole consequence of systemic hypertension unassociated with aortic dissection and each patient required aortic valve replacement. Why these 4 patients developed such severe aortic regurgitation was not determined from the operation or from necropsy. Thus, although a rare cause, systemic hypertension must be added to the list of causes of severe pure aortic regurgitation.

In past years we have studied extensively primarily by electron microscopic means porcine valve prostheses which have been used as substitute heart valves. This year we studied Bovine pericardial valve bioprostheses which had been inserted in some patients and never inserted in others. We found that pericardial bioprosthetic cusps differed from normal parietal pericardium by being denuded of mesothelium, but that they had normal degrees of waviness in their collagen. In Ionescu-Shiley valves, the inflow and outflow surfaces of each cusp corresponded to the epipericardial and serosal surfaces of parietal pericardium, respectively. The inflow surfaces had a coarse texture, characterized by large bundles of collagen, and the outflow surfaces had numerous grooves, 10-30 u in width and 20 u in depth, which probably resulted from pressure exerted on the cuspal surfaces by cotton material either during manufacturing or packing of the valves. Comparisons of the structure of bioprosthetic pericardial cusps and porcine aortic valve cusps showed that the latter had reduced degrees of collagen waviness and a different layered structure.

CONGENITAL HEART DISEASE

During a recent 4-month period we studied 2 patients who died shortly after operative "repair" of either tetralogy of Fallot or double outlet right ventricle, both with subpulmonic obstruction, and necropsy in each disclosed that a major epicardial coronary artery had been severed at operation. Because neither patient had had coronary angiography preoperatively and because death in each appeared to have resulted from the transection of the major coronary artery, a report was prepared citing the reasons for routine performance of coronary angiography preoperative in all patients with complex congenital heart disease in whom ventriculotomy is performed: 1) An operatively important coronary anomaly is sufficiently frequent in patients with many complex congenital cardiac anomalies with shunts to warrant routine coronary angiography. 2) An anomalous coronary artery coursing

across the right ventricular outflow tract in patients with cyanotic congenital heart disease may not be identifiable at the time of operation. 3) Inadvertent damage to a coronary artery at operation may have disastrous consequences in a patient with cyanotic congenital heart disease. 4) The presence of a significant coronary anomaly in a patient with cyanotic congenital heart disease may alter the timing at which a "reparative" operation is carried out and alter the type of oeprative procedure performed. 5) Coronary angiography is a safe procedure in patients with cyanotic congenital heart disease.

It is well known that patients with secundum-type atrial septal defects often have prolapse of the mitral leaflet. We studied a patient with a primum-type atrial septal defect who also had classic prolapse of the mitral leaflet and this association was never described previously. Additionally, we studied 10 patients at necropsy with Ebstein's anomaly of the tricuspid valve and three of them also had prolapse of the mitral leaflet. Thus, prolapse of the mitral valve is also common in patients with Ebstein's anomaly of the tricuspid valve as well as in patients with secundum-type atrial septal defect and rarely in patients with primum-type atrial septal defect.

MYOCARDIAL HEART DISEASE

Morphologic aspects of many cardiomyopathies in humans have been described in this unit in past years. These studies have now been extended to nonhuman animals. Findings in 15 cats 10 of whom had hypertrophic cardiomyopathy and 5 dilated cardiomyopathy, were examined. Detailed comparisons revealed many similarities to morphologic findings in humans. Thus, cardiomyopathies in the cat constitute useful models for the study of these diseases in human beings. Eleven dogs with congestive cardiomyopathy also were studied at necropsy. The ultrastructural alterations in them also were similar to those described for dilated cardiomyopathy in humans and in cats.

In past years, we studied many human hearts which have been altered by daunorubicin or adriamycin cardiotoxicity. These studies have now been extended to the rabbit. In this animal it was found that administration of an experimental antineoplastic (ICRF 187) reduces chronic daunorubicin cardiotoxicity. Chronic adriamycin was produced in 50 weanling rabbits by weekly injections of adriamycin. The lesions were similar to those which had been found earlier in humans.

It is well appreciated that exercise often brings out symtoms or abnormal physical findings which are not present at rest. Jogging or running by an estimated 25 million Americans serves as a built in exercise stress test which might uncover an underlying illness much sooner than would have been apparent had not this strenous exercise

been performed. Such was the case in 2 patients studied during the year by us both with cardiac amyloidosis. Each was a runner and their illnesses appeared to have been brought out much earlier by their stenous activity than would have been noted had not they been vigorous exercisers.

MISCELLANEOUS INVESTIGATIONS

Certain clinical and morphologic observations were described in 18 necropsy patients aged 33 to 58 years (mean 45) (14 women) with chronic hypercalcemia (11.6 to 34.4 mg/dl [19.4]) from 1 to 9 years (mean 5). Primary hyperparathyroidism was present in 9 patients and secondary hyperparathyroidism in the other 9 (of renal origin in 7). Cardiac valve anular and coronary arterial calcific deposits were present in 10 patients (group I) including 4 (mean age 51 years) with considerable narrowing of 2 or 3 of the 4 major epicardial coronary arteries. None of the other 8 patients (group II) had cardiac valve anular or cuspal calcific deposits and only 2 had coronary calcific deposits, small in each, and none had significant coronary luminal narrowing. Calcium was located in the media ("medial calcinosis"), with or without intimal deposition, of the coronary arteries in 5 patients. Comparison of the group I to the group II patients disclosed similar mean ages, durations of hypercalcemia and serum calcium levels, but signficantly ($p<.05$) higher mean total serum cholesterol levels (216-vs-163 mg/dl) and heart weights (426-vs-320 grams).

This study demonstrated that chronic hypercalcemia was associated with accelerated deposition of calcium in the cardiac anuli and valvular cusps, in the media and intima of the coronary arteries, and in individual myocardial fibers (dystrophic calcification), and that the coronary intimal calcification may be associated with or produce luminal narrowing, especially in patients in whom the serum total cholesterol level is over 200 mg/dl. Thus, chronic hypercalcemia may be viewed as a "risk factor" to accelerated coronary atherosclerosis.

Aortic dissection generally is a catastrophic cardiovascular event which in most patients is entirely preventable by proper treatment of systemic hypertension. Exactly how systemic hypertension is involved in the pathogenesis of aortic dissection is unclear, but it is clear that aortic dissection should virtually vanish if systemic hypertension disappeared. Our necropsy cases with this condition were reviewed. Patients with congenitally bicuspid aortic valves (with or without associated aortic isthmic coarctation), however, had an increased frequency of aortic dissection compared to individuals with 3 cuspid aortic valves, irrespective of the presence or absence of systemic hypertension. Most dissections involved the entire aorta. Those which stopped within the aorta with or without a reentry site generally did so because of extensive atherosclerotic plaques which caused atrophy or degeneration of the underlying media, which is where the longitudinal dissection was located. Although there are many exceptions, dissection involving the entire aorta generally indicates relatively mild or absent atherosclerosis. A common and poorly appreciated complication

of aortic dissection is stenosis of the lumen of one or more major pulmonary arteries (peripheral pulmonic stenosis) due to extension of blood from the common adventitia of the aorta to that of the pulmonary trunk. Narrowing of the lumen of the true channel of the aorta by a compressing hematoma in the false channel (true aortic stenosis) is another unappreciated but not uncommon complication of aortic dissection.

Langerhans' cells were found in lung biopsies in 1 of 9 control patients and in 20 of 160 patients with fibrotic lung disorders, including 13 of 56 patients with idiopthic pulmonary fibrosis, 2 of 9 patients with collagen vascular diseases, 2 of 7 patients with hypersensitivity pneumonitis, and each of 3 patients with end stage fibrosis of uncertain cause. Langerhans' cells were not found in any of the 41 patients with sarcoidosis, the 35 patients with interstitial lung diseases associted with inhalation of inorganic dusts, the 7 patients with pulmonary lymphangioleiomyomatosis, or the 2 patients with chronic eosinophilic pneumonia. In the control patients, Langerhans' cells were found between epithelial cells in bronchioles. In patients with fibrotic lung disorders, Langerhans' cells were found in the epithelial layer of bronchioles and alveoli containing proliferating epithelial cells, i.e., either cuboidal epithelial cells of bronchiolar origin or type II alveolar epithelial cells. Severe fibrosis or squamous metaplasia were not prerequisites for the presence of Langerhans' cells. The motility of Langerhans' cells apparently was restricted, as they were not found in the air spaces in any of the biopsies, and they were not recovered from bronchoalveolar lavage fluid of any of the 97 patients studied, even though some of these patients had relatively numerous Langerhans' cells in lung biopsies. These observations are in sharp contrast to those in pulonary histiocytosis X, in which histiocytosis X cells (HX cells) occur in granulomas, in alveolar interstititum, and between epithelial cells of the lower respiratory system. HX cells also migrate into air spaces, as shown by their occurrence in bronchoalveolar lavage fluid. The HX bodies in HX cells are morphologically similar to Langerhans' cell granules, but are more numerous and pleomorphic. HX cells are considered to be reactive or activated Langerhans' cells.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03166-01 PA

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Spontaneous exercise testing (running) as an early unmasker of underlying cardiac amyloidosis

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

PI: R.J. Siegel, Senior Staff Fellow, Pathology Branch, NHLBI

OTHER: W.J. French, Harbor UCLA Medical Center, Torrance, CA
W.C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)
Division of Cardiology, Department of Medicine, Harbor UCLA Medical Center, Torrance, California

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS: 416 hrs	PROFESSIONAL: 416 hrs	OTHER:

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

It is well appreciated that exercise often brings out symptoms or abnormal physical findings which are not present at rest. Jogging and running by an estimated 25 million Americans serves as a "built-in" exercise stress test which might uncover an underlying illness much sooner than would have been apparent had not this strenuous exercise been performed. Such appears to have been the case in 2 recently studied patients with cardiac amyloidosis.

PHS-6040
(Rev. 2-81)

Project Description: It is well appreciated that exercise often brings out symptoms or abnormal physical findings which are not present at rest. Jogging and running by an estimated 25 million Americans serves as a "built-in" exercise stress test which might uncover an underlying illness much sooner than would have been apparent had not this strenuous exercise been performed. Such appears to have been the case in 2 recently studied patients with cardiac amyloidosis. One, a 54-year-old woman, who had run approximately 14 miles weekly for a year, over a 4-month period gradually diminished the number of miles run per week because of excessive fatigue and dyspnea to the point where she was unable to run at all. Eleven months later she was dead. The second, a 46-year-old man, who had run about 12 miles weekly for 3 years, over an 8-month period gradually decreased the number of miles run per week because of excessive fatigue and dyspnea. Four months later he was dead.

In contrast to the above described patients with restrictive (amyloid) heart disease, both of whom had dyspnea and fatigue with running, patients with atherosclerotic coronary heart disease would be expected to have evidences of myocardial ischemia, namely chest pain or arrhythmia, with exercise. Thus, dyspnea and fatigue without chest pain occurring during exercise might favor non-coronary rather than coronary types of cardiac disease, as demonstrated by out above 2 patients.

Publications: Siegel, R.J., French, W.J., and Roberts, W.C.: Spontaneous exercise testing (running) as an early unmasker or underlying cardiac amyloidosis. Archives of Int Med: In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03167-01 PA

PERIOD COVERED
October 1, 1980 to September 30, 1981

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: W.C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

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INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS: 416 hrs	PROFESSIONAL: 416 hrs	OTHER:

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Certain principles of studying the heart at necropsy are summarized. 1) Do not except under quite rare circumstances, open the heart when it is fresh. After excising the heart at necropsy, fix it in formalin. 2) X-ray the heart after fixation if cardiac disease is thought to be present. Of course, postmortem clot and the crumbled bits of paper should be removed from the intact specimen before radiographs are taken. 3) The method used to open the heart is determined by the type of cardiac disease that is present or suspected.

PHS-6040
(Rev. 2-81)

Project Description: Certain principles regarding the study of the heart at necropsy have proved useful to me and they will be described in the remainder of this chapter. Do not, except under quite rare circumstances, open the heart when it is fresh. After excising the heart at necropsy, fix it in formalin or in another firming and preserving solution for at least 24 hours before opening. To preserve its shape, place small crumbled-up bits of paper (such as paper towels) loosely in the atria and in the great arteries, and, if they are dilated, in the ventricles also. Do not "stuff" the chambers for that distorts or enlarges chamber size. If the ventricles are not dilated put nothing in them, and allow the atrioventricular (A-V) valve orifices to stay as closed as possible. X-ray the heart after fixation if cardiac disease is thought to be present. Of course, postmortem clot and the crumbled bits of paper should be removed from the intact specimen before radiographs are taken. Industrial type film is ideal because contrast is far better than with the film used in live patients. Radiographs of the cardiac specimen are useful to determine the presence of and the extent of calcific deposits and to visualize the relative sizes of the cardiac chambers in proportion to the thickness of the cardiac walls. The method used to open the heart is determined by the type of cardiac disease that is present or suspected. The method most commonly used to open the heart in a patient with coronary heart disease is usually not ideal for the study of the heart of a patient with valvular or congenital heart disease.

Publications: Roberts, W.C.: Examining the Heart at Necropsy, Ch. 109. In The Heart, (Ed.) Hurst J.W., New York, McGraw-Hill (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03168-01 PA

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Aortic dissection: anatomy, consequences, and causes.

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

PI: W.C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Pathology Branch

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INSTITUTE AND LOCATION

NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Aortic dissection generally is a catastrophic cardiovascular event which in the vast majority of patients is entirely preventable by proper treatment of systemic hypertension. Exactly how systemic hypertension is involved in the pathogenesis of aortic dissection is unclear, but it is clear that aortic dissection would virtually vanish if systemic hypertension disappeared. Patients with congenitally bicuspid aortic valves (with or without associated aortic isthmic coarctation), however, have an increased frequency of aortic dissection compared to individuals with three cuspid aortic valves, irrespective of the presence or absence of systemic hypertension. Most dissections involve the entire aorta. Those which stop within the aorta with or without a reentry site generally do so because of extensive atherosclerotic plaques which may cause atrophy or degeneration of the underlying media, which is where the longitudinal dissection is located.

PHS-6040
(Rev. 2-81)

Project Description: Aortic dissection generally is a catastrophic cardiovascular event which in the vast majority of patients is entirely preventable by proper treatment of systemic hypertension. Exactly how systemic hypertension is involved in the pathogenesis of aortic dissection is unclear, but it is clear that aortic dissection whould virtually vanish if systemic hypertension disappeared. Patients with congenitally bicuspid aortic valves (with or without associated aortic isthmic coarctation), however, have an increased frequency of aortic dissection compared to individuals with three cuspid aortic valves, irrespective of the presence or absence of systemic hypertension. Most dissections involve the entire aorta. Those which stop within the aorta with or without a reentry site generally do so because of extensive atherosclerotic plaques which may cause atrophy or degeneration of the underlying media, which is where the longitudinal dissection is located. Althought there are many exceptions, dissection involving the entire aorta generally indicates relatively mild or absent atherosclerosis. A common and poorly appreciated complication of aortic dissection is stenosis of the lumen of one or more major pulmonary arteries (peripheral pulmonic stenosis) due to extension of blood from the common adventitia of the aorta to that of the pulmonary trunk. Narrowing of the lumen of the true channel of the aorta by compressing hematoma in the false channel (true aortic stenosis) is another unappreciated but not uncommon complication of aortic dissection.

Publications: Roberts, W.C.: Aortic dissection: Anatomy, consequences, and causes. Am. Heart J. 101: 195-214, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03169-01 PA

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Effect of chronic hypercalcemia on the heart. An analysis of 18 necropsy patients.

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

PI: W.C. Roberts, Chief, Pathology Branch, NHLBI

OTHER: B.F. Waller, Staff Associate, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

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Pathology Branch

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INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Certain clinical and morphologic cardiac observations are described in 18 necropsy patients aged 33 to 58 years (mean 45) (14 women) with chronic hypercalcemia (11.6 to 34.4 mg/dl [19.4]) from 1 to 9 years (mean 5). Primary hyperparathyroidism was present in 9 patients and secondary hyperparathyroidism in the other 9 (of renal origin in 7). Cardiac valve anular and coronary arterial calcific deposits were present in 10 patients (group I) including 4 (mean age 51 years) with considerable narrowing of 2 or 3 of the 4 major epicardial coronary arteries. None of the other 8 patients (group II) had cardiac valve anular or cuspal calcific deposits and only 2 had coronary calcific deposits, small in each, and none had significant coronary luminal narrowing. Calcium was located in the media ("medial calcinosis"), with or without intimal deposition, of the coronary arteries in 5 patients. Comparision of the group I to the group II patients disclosed similar mean ages, durations of hypercalcemia and serum calcium levels, but significantly ($p<.05$) higher mean total serum cholesterol levels (216-vs-163 mg/dl) and heart weights (426-vs-320 grams).

PHS-6040
(Rev. 2-81)

Project Description: Certain clinical and morphologic cardiac observations are described in 18 necropsy patients aged 33 to 58 years (mean 45) (14 women) with chronic hypercalcemia (11.6 to 34.4 mg/dl [19.4]) from 1 to 9 years (mean 5). Primary hyperparathyroidism was present in 9 patients and secondary hyperparathyroidism in the other 9 (of renal origin in 7). Cardiac valve anular and coronary arterial calcific deposits were present in 10 patients (group I) including 4 (mean age 51 years) with considerable narrowing of 2 or 3 of the 4 major epicardial coronary arteries. None of the other 8 patients (group II) had cardiac valve anular or cuspal calcific deposits and only 2 had coronary calcific deposits, small in each, and none had significant coronary luminal narrowing. Calcium was located in the media ("medial calcinosis"), with or without intimal deposition, of the coronary arteries in 5 patients. Comparison of the group I to the group II patients disclosed similar mean ages, durations of hypercalcemia and serum calcium levels, but significantly ($p<.05$) higher mean total serum cholesterol levels (216-vs-163 mg/dl) and heart weights (426-vs-320 grams).

This study demonstrates that chronic hypercalcemia is associated with accelerated deposition of calcium in the cardiac anuli and valvular cusps, in the media and intima of the coronary arteries, and in individual myocardial fibers (dystrophic calcification), and that the coronary intimal calcification may be associated with or produce luminal narrowing, especially in patients in whom the serum total cholesterol level is over 200 mg/dl. Thus, chronic hypercalcemia may be viewed as a "risk factor" to accelerated coronary atherosclerosis.

Publications: Roberts, W.C., and Waller, B.F.: Effect of chronic hypercalcemia on the heart. An analysis of 18 necropsy patients. Am J Med: In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03170-01 PA

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Prolapsing atrioventricular valve in partial atrioventricular defect.

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

PI: B.F. Waller, Staff Associate, Pathology Branch, NHLBI

OTHER: M.U. Sheikh, District of Columbia General Hospital, Washington,DC
W.C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)
District of Columbia General Hospital, Washington, D.C.

LAB/BRANCH
Pathology Branch

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INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Prolapse of the posterior mitral leaflet is now recognized to occur fairly frequently in patients with secundum-type atrial septal defect. In contrast, prolapse of an atrioventricular (AV) valve in patients with primum-type atrial septal defect (AV defect or canal) has not been reported to our knowledge. Such an occurrence, however, was observed in a woman with partial AV defect and clinical and morphologic cardiac findings were described.

PHS-6040
(Rev. 2-81)

Project number Z01 HL 03170-01 PA

Project Description: Prolapse of the posterior mitral leaflet is now recognized to occur fairly frequently in patients with secundum-type atrial septal defect. In contrast, prolapse of an atrioventricular (AV) valve in patients with primum-type atrial septal defect (AV defect or canal) has not been reported to our knowledge. Such an occurrence, however, was observed in a woman with partial AV defect and clinical and morphologic cardiac findings were described.

Publications: Waller, B.F., Sheikh, M.U., and Roberts, W.C.: Prolapsing atrioventricular valve in partial atrioventricular defect. Am Heart J. 101: 108-110, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03171-01 PA

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Ebstein's anomaly of the tricuspid valve and prolapse of the mitral valve.

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

PI: H.S. Cabin, Clinical Associate, Pathology Branch, NHLBI

OTHER: W.C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS: 416 hrs	PROFESSIONAL: 416 hrs	OTHER:

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The hearts from 10 necropsy patients with Ebstein's anomaly of the tricuspid valve were examined for abnormalities of the mitral valve. Five patients were under 1 year of age and none had an abnormal mitral valve. The other five patients were ages 18 to 72 years (mean 36 years); all had focal fibrous thickening of the mitral leaflets and three had prolapse of one or both mitral leaflets. Only one of the three patients with mitral valve prolapse had an atrial septal defect (secundum type), indicating that the association of mitral valve prolapse with Ebstein's anomaly is not due to the presence of an atrial septal defect.

PHS-6040
(Rev. 2-81)

Project number Z01 HL 03171-01 PA

Project Description: The hearts from 10 necropsy patients with Ebstein's anomaly of the tricuspid valve were examined for abnormalities of the mitral valve. Five patients were under 1 year of age and none had an abnormal mitral valve. The other five patients were ages 18 to 72 years (mean 36 years); all had focal fibrous thickening of the mitral leaflets and three had prolapse of one or both mitral leaflets. Only one of the three patients with mitral valve prolapse had an atrial septal defect (secundum type), indicating that the association of mitral valve prolapse with Ebstein's anomaly is not due to the presence of an atrial septal defect.

Publications: Cabin, H.S., and Roberts, W.C.: Ebstein's anomaly of the tricuspid valve and prolapse of the mitral valve. Am Heart J. 101:177-180, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03172-01 PA

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
The case for preoperative coronary angiography in patients with tetralogy of Fallot and other complex congenital heart diseases.

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

PI: B.M. McManus, Senior Staff Fellow, Pathology Branch, NHLBI

OTHER: B.F. Waller, Staff Associate, Pathology Branch, NHLBI
M. Jones, Surgery Branch, NHLBI
W.C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)
Surgery Branch, NHLBI

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

During a recent 4-month period we studied 2 patients who died shortly after operative "repair" of either tetralogy of Fallot or double outlet right ventricle, both with subpulmonic obstruction, and necropsy in each disclosed that a major epicardial coronary artery had been inadvertently severed at operation. Because neither patient had had coronary angiography preoperatively and because death in each appeared to have resulted from the transection of the major coronary artery, a brief summary of each patient appeared warranted as a springboard for a discussion of the rationale of coronary angiography before operative "correction" of complex congenital heart disease.

PHS-6040
(Rev. 2-81)

Project Description: During a recent 4-month period we studied 2 patients who died shortly after operative "repair" of either tetralogy of Fallot or double outlet right ventricle, both with subpulmonic obstruction, and necropsy in each disclosed that a major epicardial coronary artery had been inadvertently severed at operation. Because neither patient had had coronary angiography preoperatively and because death in each appeared to have resulted from the transection of the major coronary artery, a brief summary of each patient appeared warranted as a springboard for a discussion of the rationale of coronary angiography before operative "correction" of complex congenital heart disease.

Each of the above 2 patients demonstrate the potential or real disastrous consequences of severing a major coronary artery during a "reparative" cardiac operation for complex congenital heart disease. Neither patient preoperatively had either selective or non-selective coronary angiography and at operation the anomalous coronary artery which was severed was not identified because of either overlying epicardial adhesions or excessive adipose tissue or both. Thus, despite "successful" cardiac repair of the complex anomaly (tetralogy of Fallot or double outlet right ventricle both with subpulmonic obstruction), the presence of a simple anomaly (coronary artery) which was unidentified and inadvertently severed proved fatal in each. How can this fatal introgenic complication be prevented? The answer is routing performance of coronary angiography preoperatively in all patients with complex congenital heart disease in whom ventriculotomy is planned for the following 5 reasons: 1) An operatively important coronary anomaly is sufficiently frequent in patients with many complex congenital cardiac anomalies with shunts to warrant routine coronary angiography. 2) An anomalous coronary artery coursing across the right ventricular outflow tract in patients with cyanotic congenital heart disease may not be identifiable at the time of operation. 3) Inadvertent damage to a coronary artery at operation may have disastrous consequences in a patient with cyanotic congenital heart disease. 4) The presence of a significant coronary anomaly in a patient with cyanotic congenital heart disaese may alter the timing at which a "reparative" operation is carried out and alter the type of operative procedure performed. 5) Coronary angiography is a safe procedure in patients with cyanotic congenital heart disease.

Publications: McManus, B.M., Waller, B.F., Jones, M., and Roberts, W.C.: The case for preoperative coronary angiography in patients with tetralogy of Fallot and other complex congenital heart diseases. Am J Cardiol: In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 03173-01 PA

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Hemochromatosis heart disease: an unemphasized cause of potentially reversible restrictive cardiomyopathy.

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

PI: D.J. Cutler, Department of Medicine, Georgetown University

OTHER:
J.M. Isner, Pathology Branch, NHLBI
A.W. Bracey, Department of Surgery, Georgetown University
C.A. Hufnagel, Department of Surgery, Georgetown University
P.W. Conrad, Department of Surgery, Georgetown University
W.C. Roberts, Chief, Pathology Branch, NHLBI
D.M. Kerwin, Department of Pathology, Georgetown University
A.M. Weintraub, Department of Medicine, Georgetown University

COOPERATING UNITS (if any)
Cardiology, Surgery, and Pathology Departments, Georgetown University Hospital, Washington, D.C.

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Cardiac involvement in hemochromatosis typically results in congestive cardiomyopathy; a restrictive cardiomyopathy due to hemochromatosis is distinctly rare. A restrictive cardiomyopathy, which developed in the patient described in this report, was due to hemochromatosis which mimicked constrictive pericarditis clinically, echocardiographically and hemodynamically, and resulted in a thoracotomy for attempted surgical therapy. The fact that hemochromatosis represents the only cause of a restrictive cardiomyopathy that is potentially reversible by medical therapy makes early recognition of hemochromatosis heart disease important.

PHS-6040
(Rev. 2-81)

Project number Z01 HL 03173-01 PA

Project Description: Cardiac involvement in hemochromatosis typically results in congestive cardiomyopathy; a restrictive cardiomyopathy due to hemochromatosis is distinctly rare. A restrictive cardiomyopathy, which developed in the patient described in this report, was due to hemochromatosis which mimicked constrictive pericarditis clinically, echocardiographically and hemodynamically, and resulted in a thoracotomy for attempted surgical therapy. The fact that hemochromatosis represents the only cause of a restrictive cardiomyopathy that is potentially reverible by medical therapy makes early recognition of hemochromatosis heart disease important.

Publications: Cutler, D.J., Isner, J.M., Bracey, A.W., Hufnagel, C.A., Conrad, P.W., Roberts, W.C., Kerwin, D.M., and Weintraub, A.M.: Hemochromatosis heart disease: An unemphasized cause of potentially reversible restrictive cardiomyopathy. Am. J. Med. 69: 923-928, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03174-01 PA

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Mitral valve "anular" calcium forming a complete circle or "O" configuration: clinical and necropsy observations.

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

PI: W.C. Roberts, Chief, Pathology Branch, NHLBI

OTHER: B.F. Waller, Staff Associate, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Attention is called to the occurrence of a complete circle or "O"-shaped ring of calcium at the mitral anular region in each of two women, aged 67 and 82 years. Extension of mitral anular calcium from behind the posterior mitral leaflet across the ventricular aspect of the anterior mitral leaflet to form a complete circle has not been reported previously.

PHS-6040
(Rev. 2-81)

Project Description: Attention is called to the occurrence of a complete circle or "O"-shaped ring of calcium at the mitral anular region in each of two women, aged 67 and 82 years. Extension of mitral anular calcium from behind the posterior mitral leaflet across the ventricular aspect of the anterior mitral leaflet to form a complete circle has not been reported previously.

Publications: Roberts, W.C., and Waller, B.F.: Mitral valve "anular" calcium forming a complete circle or "O" configuration: clinical and necropsy observations. Am Heart J. 101:619, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03175-01 PA

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

M-mode echocardiographic observations in active bacterial endocarditis limited to the aortic valve: an analysis of 17 patients

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

PI: M.U. Sheikh , Department of Medicine, D.C. General Hospital

OTHER: E.A. Covarrubias , Department of Medicine, D.C. General Hospital
N. Ali , Department of Medicine, D.C. General Hospital
N.M. Sheikh , Department of Medicine, D.C. General Hospital
W.R. Lee , Department of Medicine, D.C. General Hospital
W.C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)
Cardiology Department, Georgetown and Howard University Medical Divisions, Washington, D.C. and Pathology Department, District of Columbia General Hospital, Washington, D.C.

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Analysis of 37-M-mode echocardiograms recorded during the period of active bacterial endocarditis involving the aortic valve in 17 patients disclosed 1 or more echocardiographic abnormalities involving the aortic valve cusps or their immediate vicinity in 15 (88%), including "shaggy" echos indicative of vegetations in 12 (71%). Of the 12 patients with echocardiographic evidence of aortic valve vegetation, 11 developed overt congestive heart failure and either died or had aortic valve replacement and 7 had clinical events compatible with systemic emboli; of the 5 patients without echo-demonstrated vegetations, only 1 had congestive heart failure, none had aortic valve replacement, 2 died, and 1 had a systemic embolus. In comparison to our previously reported echocardiographic observations in patients with active infective endocarditis involving either the mitral (29 patients) or tricuspid valve (23 patients), infection involving the aortic valve was far more liable to produce overt congestive heart failure and systemic emboli, to necessitate valve replacement, and to cause death during the period of active infection.

PHS-6040
(Rev. 2-81)

Project number Z01 HL 03175-01 PA

Project Description: Analysis of 37 M-mode echocardiograms recorded during the period of active bacterial endocarditis involving the aortic valve in 17 patients disclosed 1 or more echocardiographic abnormalities involving the aortic valve cusps or their immediate vicinity in 15 (88%), including "shaggy" echos indicative of vegetations in 12 (71%). Of the 12 patients with echocardiographic evidence of aortic valve vegetation, 11 developed overt congestive heart failure and either died or had aortic valve replacement and 7 had clinical events compatible with systemic emboli; of the 5 patients without echo-demonstrated vegetations, only 1 had congestive heart failure, none had aortic valve replacement, 2 died, and 1 had a systemic embolus. In comparison to our previously reported echocardiographic observations in patients with active infective endocarditis involving either the mitral (29 patients) or tricuspid valve (23 patients), infection involving the aortic valve was far more liable to produce overt congestive heart failure and systemic emboli, to necessitate valve replacement, and to cause death during the period of active infection.

Publications: Sheikh, M.U., Covarrubias, E.A., Ali, N., Sheikh, N.M., Lee, W.R., and Roberts, W.C.: M-mode echocardiographic observations in active bacterial endocarditis limited to the aortic valve: an analysis of 17 patients. Am Heart J., July 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03176-01 PA

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

M-mode echocardiographic observations during and after healing of active bacterial endocarditis limited to the mitral valve.

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

PI: M.U. Sheikh, D.C. General Hosptial

OTHER: E.A. Covarrubias, D.C. General Hospital
N. Ali, D.C. General Hospital
W.R. Lee, D.C. General Hospital
N.M. Sheikh, D.C. General Hospital
W.C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

Section of Cardiology, Georgetown and Howard University Medical Divisions, Washington, D.C. and Department of Pathology, District of Columbia General Hospital, Washington, D.C.

LAB/BRANCH

Pathology Branch

SECTION

INSTITUTE AND LOCATION

NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Analysis of 99-M-mode echocardiograms recorded during and up to 144 months after healing of active bacterial endocarditis limited to the mitral valve in 27 patients disclosed the following: 1) little to no change occurred in the echocardiographic size of the vegetations during the first 6 weeks after diagnosis and institution of appropriate antibiotic therapy unless a major systemic embolus occurred. 2) The echocardiographic size of the vegetations did not determine the amount of cardiac damage or dysfunction produced by the valvular infection. 3) The larger the vegetations by echocardiogram, the greater was the likelihood of a clinical event compatible with a systemic embolus. 4) The gravest prognostic sign yielded by the echocardiogram was evidence of rupture of chordae tendineae. 5) Although a useful adjunct to diagnosis before appropriate antibiotic therapy was instituted, once bacteriologic cure was achieved, the echocardiogram was of limited value in delineating an active from a healed vegetation. 6) The echocardiographic appearance of the vegetations was not determined by the type of infecting bacterium.

PHS-6040
(Rev. 2-81)

Project Description: Analysis of 99 M-mode echocardiograms recorded during and up to 144 months after healing of active bacterial endocarditis limited to the mitral valve in 27 patients disclosed the following: 1) little to no change occurred in the echocardiographic size of the vegetations during the first 6 weeks after diagnosis and institution of appropriate antibiotic therapy unless a major systemic embolus occurred. 2) The echocardiographic size of the vegetations did not determine the amount of cardiac damage or dysfunction produced by the valvular infection. 3) The larger the vegetations by echocardiogram, the greater was the likelihood of a clinical event compatible with a systemic embolus. 4) The gravest prognostic sign yielded by the echocardiogram was evidence of rupture of chordae tendineae. 5) Although a useful adjunct to diagnosis before appropriate antibiotic therapy was instituted, once bacteriologic cure was achieved, the echocardiogram was of limited value in delineating an active from a healed vegetation. 6) The echocardiographic appearance of the vegetations was not determined by the type of infecting bacterium.

Publications: Sheikh, M.U., Covarrubias, E.A., Ali, N., Lee, W.R., Sheikh, N.M., and Roberts, W.C.: M-mode echocardiographic observations during and after healing of active bacterial endocarditis limited to the mitral valve. Am Heart J. 101: 37, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03177-01 PA

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Postoperative Aortic Regurgitation From Incomplete Seating of Tilting-Disc Occluders Due to Overhanging Knots or Long Sutures.

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

PI: Bruce F. Waller, Staff Associate, PB, NHLBI

OTHER: Micheal Jones, Surgery Branch, NHLBI
William C. Roberts, Chief, PB, NHLBI

COOPERATING UNITS (if any)

Surgery Branch, NHLBI

LAB/BRANCH

Pathology Branch

SECTION

INSTITUTE AND LOCATION

NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Certain clinical and morphologic features are described in three patients who developed severe aortic regurgitation after aortic valve replacement. The regurgitation, fatal in two of the three patients, resulted from long sutures or their knots or both overhanging the metallic ring of the prosthesis preventing complete seating of the occluder. In one patient, simply cutting the overhanging suture eliminated the aortic regurgitation. The problem of suture overhang appears more likely to occur when aortic valve replacement is carried out in patients with relatively small aortic roots. Before closing the aortotomy after aortic valve replacement, it is advisable to move the occluder back and forth to make sure its movement, and specifically, its ability to seat completely, is not limited by sutures which are too long.

PHS-6040
(Rev. 2-81)

Project Description: Certain clinical and morphologic features are described in three patients who developed severe aortic regurgitation after aortic valve replacement. The regurgitation, fatal in two of the three patients, resulted from long sutures or their knots or both overhanging the metallic ring of the prosthesis preventing complete seating of the occluder. In one patient, simply cutting the overhanging suture eliminated the aortic regurgitation. The problem of suture overhang appears more likely to occur when aortic valve replacement is carried out in patients with relatively small aortic roots. Before closing the aortotomy after aortic valve replacement, it is advisable to move the occluder back and forth to make sure its movement, and specifically, its ability to seat completely, is not limited by sutures which are too long.

Publications: Waller, B. F., Jones, M., and Roberts, W. C.: Postoperative aortic regurgitation from incomplete seating of tilting-disc occluders due to overhanging knots or long sutures. Chest 78:565-568, October 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03178-01 PA

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Severe Aortic Regurgitation from Systemic Hypertension (without aortic dissection) Requiring Aortic Valve Replacement. Analysis of 4 Patients.

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

PI: Bruce F. Waller, Staff Associate, Pathology Branch

OTHER:
J.M. Zoltick, Department of Medicine, Georgetown University
J.H. Rosen, Department of Medicine, Georgetown University
N.M. Katz, Department of Surgery, Georgetown University
M.N. Gomes, Department of Surgery, Georgetown University
R.D. Fletcher, Department of Medicine, Georgetown University
R.B. Wallace, Department of Surgery, Georgetown University
W.C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)
Div. of Cardiology and Dept. of Surgery, Georgetown University, Washington, D.C., Department of Cardiology, Veteran's Administration Hospital, Washington, D.C.

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Clinical and morphologic observations are described in four patients who had severe aortic regurgitation from severe systemic hypertension unassociated with aortic dissection and each patient underwent aortic valve replacement. Although aortic regurgitation of minimal or mild degree is well recognized to occur in patients with systemic hypertension, severe degrees of aortic requrgitation in such patients is rare and aortic valve replacement in such patients has not been reported previously.

PHS-6040
(Rev. 2-81)

Project Description: Clinical and morphologic observations are described in four patients who had severe aortic regurgitation from severe systemic hypertension unassociated with aortic dissection and each patient underwent aortic valve replacement. Although aortic regurgitation of minimal or mild degree is well recognized to occur in patients with systemic hypertension, severe degrees of aortic regurgitation in such patients is rare and aortic valve replacement in such patients has not been reported previously. Why our four patients developed such severe aortic regurgitation, however, was not determined. Although a rare cause, systemic hypertension, nevertheless, must be added to the list of causes of severe pure aortic regurgitation.

Publications: Waller, B.F., Zoltick, J.M., Rosen J.H., Katz, N.M., Gomes, M.N., Fletcher, R.D., Wallace, R.B., and Roberts, W.C.: Severe aortic regurgitation from systemic hypertension (without aortic dissection) requiring aortic valve replacement. Analysis of 4 patients. Am J Cardiol.: In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03179-01 PA

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Comparison of amount and extent of coronary narrowing by atherosclerotic plaque and of myocardial scarring at necropsy in anterior-vs-posterior healed transmural myocardial infarction.

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

PI: H.S. Cabin, Clinical Associate, Pathology Branch, NHLBI

OTHER: W.C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The amount of cross-sectional area narrowing by atherosclerotic plaque in each 5-mm segment from the entire lengths of the right, left anterior descending and left circumflex coronary arteries and the size, predominant location and extent of myocardial scarring were determined in 59 necropsy with a healed transmural infarct (MI). The mean number of the 4 major epicardial coronary arteries narrowed severely (76-100% in cross-sectional area) was 3.0 in the 37 patients with posterior MI and 2.6 in the 22 patients with anterior MI ($p < .025$). The mean percent of 5-mm segments narrowed severely from all 4 major coronary arteries was similar in the anterior and posterior MI groups (38%-vs-46%). The patients with anterior MI, however, had a higher percentage of 5-mm segements of the left anterior descending severely narrowed than of the left circumflex but not the right coronary artery (46%-vs-25% [$p < .001$] and 40% [ns]). The patients with posterior MI had a higher percentage of segments of the right and left circumflex coronary arteries severely narrowed than of the left anterior desceding (55% and 51%-vs-32%) ($p < .05$).

PHS-6040
(Rev. 2-81)

Project Description: The amount of cross-sectional area narrowing by atherosclerotic plaque in each 5-mm long segment from the entire lengths of the right, left main, left anterior descending and left circumflex coronary arteries and the size, predominant location and extent of myocardial scarring were determined in 59 necropsy patients with a healed transmural myocardial infarct (MI). The mean number of the 4 major epicardial coronary arteries narrowed severely (76-100% in cross-sectional area) was 3.0 in the 37 patients with posterior MI and 2.6 in the 22 patients with anterior MI ($p<.025$). The mean percent of 5-mm segments narrowed severely from all 4 major coronary arteries was similar in the anterior and posterior MI groups (38%-vs-46%). The patients with anterior MI, however, had a higher percentage of 5-mm segments of the left anterior descending severely narrowed than of the left circumflex but not the right coronary artery (46%-vs-25% [$p<.001$] and 40% [ns]). The patients with posterior MI had a higher percentage of segments of the right and left circumflex coronary arteries severely narrowed than of the left anterior descending (55% and 51%-vs-32%) ($p<.05$). The anterior MI group had on the average larger left ventricular scars than the posterior MI group (20%-vs-9%), ($p<.002$) and more frequent scarring of the ventricular septum (16[73%] patients - vs - 6[16%]) ($p<.001$).

Publications: Cabin, H.S., and Roberts, W.C.: Comparison of amount and extent of coronary narrowing by atherosclerotic plaque and of myocardial scarring at necropsy in anterior-vs-posterior healed transmural myocardial infarction. Circulation: In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03180-01 PA

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Type III hyperlipoproteinemia: quantification of amounts of coronary arterial narrowing in 5 necropsy patients.

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

PI: H.S. Cabin, Clinical Associate, Pathology Branch, NHLBI

OTHER: D.E. Schwartz, Molecular Disease Branch, NHLBI
R. Virmani, Pathology Branch, NHLBI
H.B. Brewer, Molecular Disease Branch, NHLBI
W.C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

Molecular Disease Branch, NHLBI

LAB/BRANCH

Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS: 416 hrs	PROFESSIONAL: 416 hrs	OTHER:

CHECK APPROPRIATE BOX(ES)

[x] (a) HUMAN SUBJECTS [x] (b) HUMAN TISSUES [ ] (c) NEITHER

[ ] (a1) MINORS [ ] (a2) INTERVIEWS

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

The amount of cross-sectional area narrowing in each 5-mm long segment of each of the 4 major epicardial coronary arteries was determined in each of 5 patients with type III hyperlipoproteinemia and symptomatic, fatal atherosclerotic coronary heart disease. Four had had angina pectoris; 2, acute myocardial infarcts which healed, and 2 died suddenly. Of the 4 major epicardial coronary arteries, all 4 were narrowed 76-100% in cross-sectional area by atherosclerotic plaques in 2 patients, 3 were narrowed to this degree in 2 patients and 2 were so narrowed in 1 patient. Three patients had severe narrowing of the left main coronary artery. The percent of 5-mm long segments of coronary artery narrowed to various degrees was as follows: 96-100%, 0-37 (mean 14); 76-95%, 14-61 (mean 35); 51-75%, 9-41 (mean 24); 26-50%, 0-42 (mean 16), and 0-25%, 0-27 (mean 11). Utilizing a scoring system of 1 to 4 for the 4 categories of narrowing (1=0-25%, 2=26-50%, 3=51-75% and 4=76-100% cross-sectional area narrowing), the scores per 5-mm segment for each patient ranged from 2.5 - 3.9 (mean 3.1).

PHS-6040
(Rev. 2-81)

Project Description: The amount of cross-sectional area narrowing in each 5-mm long segment of each of the 4 major epicardial coronary arteries was determined in each of 5 patients with type III hyperlipoproteinemia and symptomatic, fatal atherosclerotic coronary heart disease. Four had had angina pectoris; 2, acute myocardial infarcts which healed, and 2 died suddenly. Of the 4 major epicardial coronary arteries, all 4 were narrowed 76-100% in cross-sectional area by atherosclerotic plaques in 2 patients, 3 were narrowed to this degree in 2 patients and 2 were so narrowed in 1 patient. Three patients had severe narrowing of the left main coronary artery. The percent of 5-mm long segments of coronary artery narrowed to various degrees was as follows: 96-100%, 0-37 (mean 14); 76-95%, 14-61 (mean 35); 51-75%, 9-41 (mean 24); 26-50%, 0-42 (mean 16), and 0-25%, 0-27 (mean 11). Utilizing a scoring system of 1 to 4 for the 4 categories of narrowing (1=0-25%, 2=26-50%, 3=51-75% and 4=76-100% cross-sectional area narrowing), the scores per 5-mm segment for each patient ranged from 2.5 - 3.9 (mean 3.1). Thus, these 5 type III patients had severe diffuse coronary narrowing by atherosclerotic plaques.

Publications: Cabin, H.S., Schwartz, D.E., Virmani, R., Brewer, H.B., and Roberts, W.C.: Type III hyperlipoproteinemia: quantification of amounts of coronary arterial narrowing in 5 necropsy patients. Amer Heart J: In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03181-01 PA

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Survival for 20 years or longer after tarnsmural acute myocardial infarction: an analysis of 8 well-documented necropsy patients.

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

PI: B.M. McManus, Senior Staff Fellow, Pathology Branch, NHLBI

OTHER: W.C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

[X] (a) HUMAN SUBJECTS [X] (b) HUMAN TISSUES [ ] (c) NEITHER

[ ] (a1) MINORS [ ] (a2) INTERVIEWS

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

Clinical and necropsy findings are described in 8 patients who lived 20-31 years (mean 24) after healing of a transmural acute myocardial infarct. Two had left ventricular aneurysms and one had both right and left ventricular infarcts. Survival for 2 decades or more after healing of a transmural acute myocardial infarct has rarely been documented and descriptions of hearts at necropsy in patients with well-documented infarcts 20 years or more earlier are virtually nonexistent.

PHS-6040
(Rev. 2-81)

Project number Z01 HL 03181-01 PA

Project Description: Clinical and necropsy findings are described in 8 patients who lived 20-31 years (mean 24) after healing of a transmural acute myocardial infarct. Two had left ventricular aneurysms and one had both right and left ventricular infarcts. Survival for 2 decades or more after healing of a transmural acute myocardial infarct has rarely been documented and descriptions of hearts at necropsy in patients with well-documented infarcts 20 years or more earlier are virtually nonexistent.

Publications: McManus, B.M., and Roberts, W.C.: Survival for 20 years or longer after transmural acute myocardial infarction: an analysis of 8 well-documented necropsy patients. Amer Heart J: In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03182-01 PA

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Fatal cardiac arrest during cardiac catheterization for angina pectoris: an analysis of 10 necropsy patients.

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

PI: H.S. Cabin, Clinical Associate, Pathology Branch, NHLBI

OTHER: W.C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Ten patients with angina pectoris and fatal cardiac arrest during cardiac catheterization were analyzed to determine the circumstances of death and the severity and distribution of the coronary arterial narrowing. Nine patients died during attempted coronary angiography, and the remaining patient, during right-sided cardiac catheterization. All 10 patients had at least three of four major epicardial coronary arteries narrowed 76-100% in cross-sectional area by atherosclerotic plaques, including seven with this degree of narrowing of the left main coronary artery by plaque; two additional patients had severe (>75%) narrowing of the left main coronary artery by thromboembolic material superimposed on small atherosclerotic plaques. Of 354 five-mm long segments of the left main, left anterior descending, left circumflex and right coronary arteries in eight patients, the percent narrowed to various degrees in cross-sectional area by atherosclerotic plaques was as follows: 96-100% = 9; 51-75% = 23; 26-50% = 13, and 0-25% = 6.

PHS-6040
(Rev. 2-81)

Project Description: Ten patients with angina pectoris and fatal cardiac arrest during cardiac catheterization were analyzed to determine the circumstances of death and the severity and distribution of the coronary arterial narrowing. Nine patients died during attempted coronary angiography, and the remaining patient, during right-sided cardiac catheterization. All 10 patients had at least three of four major epicardial coronary arteries narrowed 76-100% in cross-sectional area by atherosclerotic plaques, including seven with this degree of narrowing of the left main coronary artery by plaque; two additional patients had severe (>75%) narrowing of the left main coronary artery by thromboembolic material superimposed on small atherosclerotic plaques. Of 354 five-mm long segments of the left main, left anterior descending, left circumflex and right coronary arteries in eight patients, the percent narrowed to various degrees in cross-sectional area by atherosclerotic plaques was as follows: 96-100% = 9; 76-95% = 49; 51-75% = 23; 26-50% = 13, and 0-25% = 6. Utilizing a scoring system of one to four for the amount of narrowing in each five-mm segment (one = 0-25%, two = 26-50%, three = 51-75%, four = 76-100%), the mean score per five-mm segment for the group was 3.34. Thus, patients with angina pectoris who die during cardiac catheterization have particularly severe and diffuse coronary atherosclerosis and usually severe narrowing of the left main coronary artery.

Publications: Cabin, H.S., and Roberts, W.C.: Fatal cardiac arrest during cardiac catheterization for angina pectoris: an analysis of 10 necropsy patients. Am J Cardiol. 48: 1-8, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03183-01 PA

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Amount of narrowing by atherosclerotic plaque in 44 nonbypassed and 52 bypassed major epicardial coronary arteries in 32 necropsy patients who died within 1 month of aortocoronary bypass grafting.

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

PI: B.F. Waller, Staff Associate, Pathology Branch, NHLBI

OTHER: W.C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

In 32 necropsy patients who died within 30 days of an aortocoronary bypass operation performed for relief of angina pectoris, the lumens in 42 (95%) of 44 nonbypassed and in 52 (100%) of 52 bypassed arteries were narrowed 76 to 100% in cross-sectional area by atherosclerotic plaque. Of 616 five mm segments of the 44 nonbypassed arteries examined histologically, 292 (47%) were narrowed 76 to 100% in cross-sectional area by atherosclerotic plaque; of 728 segments examined in the 52 bypassed arteries, 375 (52%) were similarly narrowed. Thirty-two (73%) of the 44 nonbypassed coronary arteries (in 23 patients) had been judged to be narrowed 50% or less in diameter on preoperative coronary angiography, but at necropsy 31 (97%) of these arteries were narrowed 76 to 100% in cross-sectional area and the other artery was narrowed 51 to 75%.

PHS-6040
(Rev. 2-81)

Project number Z01 HL 03183-01 PA

Project description: In 32 necropsy patients who died within 30 days of an aortocoronary bypass operation performed for relief of angina pectoris, the lumens in 42 (95%) of 44 nonbypassed and in 52 (100%) of 52 bypassed arteries were narrowed 76 to 100% in cross-sectional area by atherosclerotic plaque. Of 616 five mm segments of the 44 nonbypassed arteries examined histologically, 292 (47%) were narrowed 76 to 100% in cross-sectional area by atherosclerotic plaque; of 728 segments examined in the 52 bypassed arteries, 375 (52%) were similarly narrowed. Thirty-two (73%) of the 44 nonbypassed coronary arteries (in 23 patients) had been judged to be narrowed 50% or less in diameter on preoperative coronary angiography, but at necropsy 31 (97%) of these arteries were narrowed 76 to 100% in cross-sectional area and the other artery was narrowed 51 to 75%. Thus, significant amounts of atherosclerotic plaque tend to be present at necropsy in all three major coronary systems of patients with angina pectoris who die early after an aortocoronary bypass operation.

Publications: Waller, B.F., and Roberts, W.C.: Amount of narrowing by atherosclerotic plaque in 44 nonbypassed and 52 bypassed major epicardial coronary arteries in 32 necropsy patients who died within 1 month of aortocoronary bypass grafting. Am J Cardiol. 46: 956-962, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03184-01 PA

PERIOD COVERED
October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Ultrastructural Evaluation of Porcine Valvular Bioprostheses

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

PI:	Victor J. Ferrans, Chief, Ultrastructure Section	PA	NHLBI
Others:	S. W. Boyce, Ultrastructure Section, Pathology Br.	PA	NHLBI
	M. E. Billingham, Assistant Professor, Pathology Department, Stanford Univ. Medical Center		
	Michael Jones, Senior Surgeon, Surgery Branch	SU	NHLBI
	Thomas L. Spray, Pathology Branch	PA	NHLBI
	W. C. Roberts, Chief, Pathology Branch	PA	NHLBI

COOPERATING UNITS (if any)

Surgery Branch, NHLBI
Dept. Pathology, Stanford Univ. Med. Center, Stanford, Cal.

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.5	PROFESSIONAL: 0.4	OTHER: 0.1

CHECK APPROPRIATE BOX(ES)

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

☐XX (a1) MINORS ☐ (a2) INTERVIEWS

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

A description is presented of structural alterations found in 30 porcine aortic valvular bioprostheses that had been implanted in patients.

PHS-6040
(Rev. 2-81)

Project Description: Structural alterations were studied in 30 porcine valvular bioprostheses removed from 28 patients either at necropsy (12 patients) or at reoperation for replacement of a malfunctioning bioprosthesis (16 patients). Early changes (< 2 months after implantation) consisted of deposition of fibrin, macrophages and multinucleated giant cells on the valvular surfaces; insudation of plasma proteins into the valvular substance, and breakdown of collagen in superficial regions of the leaflets. Late changes (3 to 94 months after implantation) were characterized by progressively severe damage to the collagen, aggregation of platelets on the surfaces, lipid deposition, surface erosion and calcification. Perforation of the leaflets, leading to prosthetic regurgitation, was a consequence of severe collagen breakdown. Prosthetic stenosis most frequently resulted from calcification of valvular collagen. Thus, alterations in collagen are of crucial importance in determining the long-term durability of porcine valvular bioprostheses.

Publications: Ferrans, V. J., Boyce, S. W., Billingham, M. E., Jones, M., Spray, T. L. and Roberts, W. C.: Ultrastructural evaluation of porcine valvular heterografts. In: Selected Topics in Cardiac Surgery. Proceedings of the Symposium held in Padova, Italy, May 25-26, 1979. V. Gallucci, R. M. Bini, and G. Thiene (Eds.), Patron Editore Bologna, 1980, pp. 225-239.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03185-01 PA

PERIOD COVERED

October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Pathology of Saphenous Vein Aortocoronary Bypass Grafts

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

PI:	Victor J. Ferrans, Chief, Ultrastructure Section	PA	NHLBI
Others:	Michael Jones, Senior Surgeon, Surgery Branch	SU	NHLBI
	W. C. Roberts, Chief, Pathology Branch	PA	NHLBI

COOPERATING UNITS (if any)

Surgery Branch

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.3	PROFESSIONAL: 0.2	OTHER: 0.1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

A review is presented of gross anatomic, histologic and ultrastructural changes occurring in saphenous vein aortocoronary bypass grafts after implantation.

PHS-6040
(Rev. 2-81)

Project Description: A review is presented of the gross anatomic, histologic and ultrastructural changes occurring in aortocoronary bypass graft systems. These changes are discussed according to whether they are related to: 1) problems of operative judgement and technique; 2) tissue responses of the vein to the implantation procedure and subsequent hemodynamic changes, and 3) alterations involving the coronary arteries proximal and distal to the graft sites.

Publications: Ferrans, V. J., Jones, M., and Roberts, W. C.: The pathology of saphenous vein aortocoronary bypass grafts. In: Selected Topics in Cardiac Surgery. Proceedings of the Symposium held in Padova, Italy, May 25-26, 1979. V. Gallucci, R. M. Bini, and G. Thiene (Eds.), Patron Editore Bologna, 1980, pp. 423-439.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03186-01 PA

PERIOD COVERED
October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Structure of Bovine Pericardium and Unimplanted Pericardial Bioprostheses

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

PI:	Victor J. Ferrans, Chief, Ultrastructure Section	PA	NHLBI
Others:	Tokuhiro Ishihara, Guest Worker, Ultrastructure Section	PA	NHLBI
	Michael Jones, Senior Surgeon, Surgery Branch	SU	NHLBI
	Steven W. Boyce, Ultrastructure Section	PA	NHLBI
	W. C. Roberts, Chief, Pathology Branch	PA	NHLBI

COOPERATING UNITS (if any)
Surgery Branch, NHLBI

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.5	PROFESSIONAL: 0.4	OTHER: 0.1

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

To obtain a basis for the evaluation of postimplantation changes in bioprostheses made of parietal pericardium, comparative histologic, scanning and transmission electron microscopic studies were made of the structure of: 1) normal bovine parietal pericardium; 2) glutaraldehyde-treated pericardial patches to be used for repair of cardiac defects, and 3) pericardial tissue cusps of unimplanted Ionescu-Shiley valves.

PHS-6040
(Rev. 2-81)

Project Description: To obtain a basis for the evaluation of post-implantation changes in bioprostheses made of parietal pericardium, comparative histologic, scanning and transmission electron microscopic studies were made of the structure of: 1) normal bovine parietal pericardium; 2) glutaraldehyde-treated pericardial patches to be used for repair of cardiac defects, and 3) pericardial tissue cusps of unimplanted Ionescu-Shiley valves. Bovine parietal pericardium has three layers: 1) the serosa, or mesothelial cell layer; 2) the fibrosa, formed by diversely oriented, wavy bundles of collagen and by elastic fibers, and 3) the epipericardial connective tissue layer, which is partly continuous with the pericardiosternal ligaments. Pericardial patches and pericardial bioprosthetic cusps differ from normal pericardium by being denuded of mesothelium, but have normal degrees of waviness in their collagen. In Ionescu-Shiley valves, the inflow and outflow surfaces of each cusp correspond to the epipericardial and serosal surfaces of parietal pericardium, respectively. The inflow surfaces have a coarse texture, characterized by large bundles of collagen, and the outflow surfaces have numerous grooves, 10-30 μ in width and 20 μ in depth, which probably result from pressure exerted on the cuspal surfaces by cotton material either during manufacturing or packing of the valves. Comparisons of the structure of bioprosthetic pericardial cusps and porcine aortic valve cusps show that the latter have reduced degrees of collagen waviness and a different layered structure: a layer similar to the spongiosa of aortic valve cusps is not present in pericardium. The functional implications of these observations are discussed in detail.

Publications: Ishihara, T., Ferrans, V. J., Jones, M., Boyce, S. W. and Roberts, W. C.: Structure of bovine parietal pericardium and of unimplanted Ionescu-Shiley pericardial valvular bioprostheses. J. Thorac. Cardiovasc. Surg. 81: 747-757, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03187-01 PA

PERIOD COVERED
October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Pathologic Alterations in Congestive Cardiomyopathy of Dogs

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

PI: Victor J. Ferrans, Chief, Ultrastructure Section PA NHLBI

Others: John Van Vleet, Professor, Department of Microbiology, Pathology and Public Health, School of Veterinary Medicine, Purdue University

Walter E. Weirich, Dept. of Small Animal Clinics, School of Veterinary Medicine, Purdue University

COOPERATING UNITS (if any)

School of Veterinary Medicine, Purdue University, West Lafayette, Ind.

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.1	PROFESSIONAL: 0.1	OTHER:

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Detailed comparisons reveal many similarities in the spectrum of morphologic features of congestive cardiomyopathy in humans and in large breed dogs (most frequently, male Great Danes)

PHS-6040
(Rev. 2-81)

Project Description: Eleven large-breed dogs with congestive cardiomyopathy were studied at necropsy. Seven of 11 dogs were Great Danes and 9 of 11 were males. The most common clinical signs in affected dogs were dyspnea, abdominal distention by ascites, and weight loss. Three dogs had severe congestive cardiac failure. At necropsy, gross alterations in the hearts were cardiomegaly, dilatation of all chambers, opacity of the endocardium, atrial thrombosis, and disseminated foci of myocardial necrosis. Extracardiac findings included hepatic congestion, pulmonary congestion and edema, ascites, and infarction of kidney, liver, lung and spleen. Histologically, the affected hearts had disseminated foci of subendocardial myocardial necrosis, scattered areas of myocardial fibrosis and medial hyperplasia of intramyocardial arteries. Ultrastructurally, degenerated cardiac muscle cells had myocytolysis, proliferated elements of sarcoplasmic reticulum, numerous myelin figures and autophagic vacuoles, increased numbers of lipofuscin granules and mitochondrial alterations. These ultrastructural alterations are similar to those described for cardiomyopathy in humans and cats.

Publications: Van Vleet, J. F., Ferrans, V. J. and Weirich, W. E.: Pathologic alterations in congestive cardiomyopathy in dogs. Am. J. Vet. Res. 42: 416-424, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03188-01 PA

PERIOD COVERED
October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Reduction of Daunorubicin Cardiotoxicity by ICRF 187 in Rabbits

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

PI: Victor J. Ferrans, Chief, Ultrastructure Section PA NHLBI
Others: Eugene H. Herman, Division of Drug Biology, FDA
William Jordan, Division of Drug Biology, FDA
Bach Ardalan, Division of Drug Biology, FDA

COOPERATING UNITS (if any)

FDA, Division of Drug Biology

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.2	PROFESSIONAL: 0.2	OTHER:

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The chronic cardiotoxicity produced in rabbits by daunorubicin, an antineoplastic agent of great clinical importance in human medicine, was found to be greatly reduced by the concurrent administration of ICRF 187, the d-isomer of ICRF 159 (1,2-bis(3,5-dioxopiperazinyl-1-yl) propane).

PHS-6040
(Rev. 2-81)

Project Description: To determine whether ICRF-187 (NSC-169780) would alter chronic daunorubicin (NSC-82151) cardiac toxicity, male New Zealand rabbits were given 3.2 mg/kg of daunorubicin iv alone or 30 minutes after 12.5 or 25.0 mg/kg of ICRF-187 ip at 3-week intervals. Control rabbits received either saline iv or ICRF-187 (12.5 or 25.0 mg/kg) ip on the same schedule. Three weeks after the fifth injection, the animals were sacrificed. The frequency and extent of cellular alterations were graded on a scale of 0 to 4. Lesions consisting mainly of vacuolization and myofibrillar loss were noted in the hearts of all 12 rabbits given daunorubicin alone. The severity ranged from 1 to 3 (average 1.8). In contrast, no abnormalities were noted in one of five (12.5 mg/kg) and three of seven (25.0 mg/kg) ICRF-treated rabbits. The remaining eight hearts from both pretreatment groups displayed minimal alterations ranging from 0.5 to 1.0 (average 0.9). Thus, concurrent administration of the antineoplastic agent ICRF-187 may offer a means of reducing chronic daunorubicin cardiac toxicity.

Publications: Herman, E. H., Ferrans, V. J., Jordan, W. and Ardalan, B.: Reduction of chronic daunorubicin cardiotoxicity by ICRF-187 in rabbits. Res. Commun. Chem. Pathol. Pharmacol. 31: 85-97, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03189-01 PA

PERIOD COVERED
October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Selenium-vitamin E Deficiency in Swine Fed Various Metals

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

PI: Victor J. Ferrans, Chief, Ultrastructure Section PA NHLBI
Others: John F. Van Vleet, Professor, Department of Microbiology, Pathology and Public Health, School of Veterinary Medicine, Purdue University
G. Daniel Boon, Department of Microbiology, Pathology and Public Health, School of Veterinary Medicine, Purdue University

COOPERATING UNITS (if any)

School of Veterinary Medicine, Purdue University, West Lafayette, Ind.

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.1	PROFESSIONAL: 0.1	OTHER:

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Evidence of selenium-vitamin E deficiency, as indicated by typical microscopic findings in cardiac and skeletal muscle, was found in 50 to 65% of pigs fed cobalt or tellurium and occasionally in pigs fed zinc, cadmium or vanadium supplements.

PHS-6040
(Rev. 2-81)

Project Description: Forty-two weanling pigs were allotted to 7 groups and fed (for 10 weeks) a commercial ration that was adequate in selenium and vitamin E (Se-E) content, either alone or with supplements of Ag (3,000 mg/kg of feed, as acetate), Co (500 mg/kg, as chloride), Te (500 mg/kg, as tetrachloride), Zn (3,000 mg/kg, as sulfate), Cd (500 mg/kg, as sulfate), or V (200 mg/kg, as vanadate). The pigs fed the Ag supplement died after 25 to 39 days and had lesions characteristic of Se-E deficiency with accumulations of serous transudates in body cavities and hepatic and cardiac necrosis. In the pigs fed the Ag supplement, there was high hepatic Se content terminally; blood glutathione peroxidase (GSH-Px) activity decreased to low levels several weeks before the pigs died with lesions of Se-E deficiency. Macroscopic lesions of Se-E deficiency were not found in pigs fed Co, Te, Zn, Cd, or V. However, evidence of Se-E deficiency, as indicated by microscopically detected necrosis of cardiac and skeletal muscle, was present in 50% to 65% of the pigs fed Co or Te and occasionally in pigs fed Zn, Cd, and V supplements.

The pigs fed Te had marked decrease of blood GSH-Px activity over the last 6 weeks of the feeding period. No consistently abnormal values for blood GSH-Px activity or terminal hepatic Se content were observed in pigs fed Co, Zn, Cd, or V. The pigs fed the Zn supplement grew as rapidly as the control pigs. Evidence of V toxicosis was observed as severe growth suppression, mortality, and marked enteritis and cystitis (with accompanying hydroureter in 1 pig).

Publications: Van Vleet, J. F., Boon, G. D. and Ferrans, V. J.: Induction of lesions of selenium-vitamin E deficiency in weanling swine fed silver, cobalt, tellurium, zinc, cadmium, and vanadium. Am. J. Vet. Res. 42: 789-799, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03190-01 PA

PERIOD COVERED
October 1, 1980, to September 30, 1981

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	PA	NHLBI
Others:	W. C. Roberts, Chief, Pathology Branch	PA	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.05	PROFESSIONAL: 0.05	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 review is presented of cardiac morphologic changes that occur in acute rheumatic fever and in chronic rheumatic heart disease.

PHS-6040
(Rev. 2-81)

Project Description: Acute rheumatic fever is manifested by inflammation of the endocardium, myocardium and pericardium; this inflammation often is associated with extracardiac lesions that involve the aorta, coronary and pulmonary arteries, the joints, the skin, the lungs and the central nervous system. Cardiac lesions in chronic rheumatic heart disease consist of valvular deformities which cause valvular and myocardial dysfunction. The gross anatomic, histologic and ultrastructural features of these acute and chronic changes are reviewed in detail.

Publications: Ferrans, V. J., and Roberts, W. C.: Pathology of rheumatic heart disease. In: Rheumatic Valvular Disease in Children. J. B. Borman and M. S. Gotsman (Eds.), Springer-Verlag, Berlin, Heidelberg, New York, 1980, pp. 28-58.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03191-01 PA

PERIOD COVERED
October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Cardiotoxicity of Adriamycin in Rabbits

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

PI: Victor J. Ferrans, Chief, Ultrastructure Section PA NHLBI
Other: John Van Vleet, Professor, Department of Microbiology, Pathology and Public Health, School of Veterinary Medicine, Purdue University

COOPERATING UNITS (if any)

School of Veterinary Medicine, Purdue University, West Lafayette, Indiana

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.05	PROFESSIONAL: 0.05	OTHER:

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The clinical and pathologic manifestations of the chronic cardiotoxicity produced in rabbits by the administration of adriamycin are described.

PHS-6040
(Rev. 2-81)

Project Description: Chronic adriamycin (ADR) intoxication was produced in 50 weanling rabbits by weekly injections of ADR (2.4 mg/kg of body weight) for up to 17 weeks. All ADR-treated rabbits developed prominent alopecia, edema, moderate anemia, and severe emaciation. The frequency and severity of cardiomyopathy were not strongly dose-related, but renal lesions were. The frequency and severity of cardiomyopathic lesions were highest in left ventricular free wall and ventricular septum, intermediate in atria, and lowest in right ventricular free wall. Cardiac lesions increased in frequency and severity basally in left ventricular free wall, ventricular septum, and atria, but not in right ventricular free wall. Nephropathy lesions were selective for inner cortex and constitute a common and important side effect of chronic ADR intoxication in rabbits. Other lesions observed at necropsy were hepatic necrosis, skeletal muscle degeneration, osteodystrophy-associated fractures, peripancreatic fat necrosis, and testicular degeneration and atrophy.

Publications: Van Vleet, J. F. and Ferrans, V. J.: Clinical and pathologic features of chronic adriamycin toxicosis in rabbits. Am. J. Vet. Res. 41: 1462-1469, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03192-01 PA

PERIOD COVERED
October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Pathologic Alterations in Hypertrophic and Congestive Cardiomyopathy in Cats

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

PI: Victor J. Ferrans, Chief, Ultrastructure Section PA NHLBI

Others: John Van Vleet, Professor, Department of Microbiology, Pathology and Public Health, School of Veterinary Medicine, Purdue University

Walter E. Weirich, Dept. of Small Animal Clinics, School of Veterinary Medicine, Purdue University

COOPERATING UNITS (if any)

School of Veterinary Medicine, Purdue University, West Lafayette, Ind.

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.05	0.05	

CHECK APPROPRIATE BOX(ES)

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Detailed comparisons reveal many similarities in the spectrum of morphologic features of hypertrophic cardiomyopathy and congestive cardiomyopathy in humans and cats. It is concluded that cardiomyopathies in the cat constitute useful models for the study of these diseases in humans.

PHS-6040
(Rev. 2-81)

Project Description: Pathologic alterations were studied in 15 cats with cardiomyopathy. Ten of these had hypertrophic cardiomyopathy, characterized by cardiomegaly, diffuse myocardial hypertrophy, small left ventricular cavities, and dilated left atria; the ventricular septum and left ventricular free walls were thick, but asymmetric septal hypertrophy was not present. Aortic thromboembolism, renal infarction, and pulmonary congestion and edema were frequent.

Histopathologic and ultrastructural study revealed hypertrophy and disarray of cardiac muscle cells (most severe in left ventricle and ventricular septum), interstitial fibrosis, and fibromuscular hyperplasia of small intramural coronary arteries. The hypertrophied fibers had large nuclei, prominent Golgi complexes, and numerous polysomes; some fibers had crisscrossing myofibrils. Degenerative alterations in hypertrophied myocytes were: perinuclear distension of elements of sarcoplasmic reticulum, focal myofibrillar lysis, numerous thick clumps of Z-band material, and abundant lipofuscin granules. The interstitium showed accumulations of collagen fibrils, increased numbers of fibroblasts, and scattered remnants of external lamina. One cat had dilated chambers, but also had typical histologic and ultrastructural alterations of hypertrophic cardiomyopathy and was considered to have a late stage of this disorder. Four cats had congestive cardiomyopathy, with cardiomegaly and dilatation of all chambers. Hydrothorax and pulmonary congestion and edema were generally present. Cardiac histopathologic and ultrastructural alterations consisted only of mild interstitial edema and fibrosis.

Detailed comparisons reveal many similarities in the spectrum of morphologic features of hypertrophic cardiomyopathy in human beings and cats; there are not marked differences in the morphologic features of congestive cardiomyopathy in human beings and cats. Thus, cardiomyopathies in the cat constitute useful models for the study of these diseases in human beings.

Publications: Van Vleet, J. F., Ferrans, V. J. and Weirich, W. E.: Pathologic alterations in hypertrophic and congestive cardiomyopathy of cats. Am. J. Vet. Res. 41: 2037-2048, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03193-01 PA

PERIOD COVERED
October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Early Calcification of a Pericardial Bioprosthetic Valve

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

PI:	Victor J. Ferrans, Chief, Ultrastructure Section	PA	NHLBI
Others:	Tokuhiro Ishihara, Guest Worker, Ultrastructure Section	PA	NHLBI
	Michael Jones, Senior Surgeon, Surgery Branch	SU	NHLBI
	Henry S. Cabin, Clinical Associate,	PA	NHLBI
	W. C. Roberts, Chief, Pathology Branch	PA	NHLBI

COOPERATING UNITS (if any)

Surgery Branch, NHLBI

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.05	PROFESSIONAL: 0.05	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)

Structural studies were made of calcific deposits found 3 days after implantation in a pericardial bioprosthetic valve. The deposits were localized on thrombi covering the cuspal surfaces.

PHS-6040
(Rev. 2-81)

Project Description: Calcific deposits, localized in a thin layer of thrombus covering the cuspal surfaces, were present 3 days after implantation in a valved pulmonic conduit that contained a Ionescu-Shiley bovine pericardial valve and was placed in a 29-year-old man with double outlet right ventricle, valvular and infundibular pulmonic stenosis, and ventricular septal defect. Factors that may have contributed to such a rapid calcification were the relatively young age of the patient, the development of acute renal insufficiency postoperatively, and the administration of large amounts of calcium chloride intravenously during blood transfusions and during episodes of cardiac arrest.

Publication: Ishihara, T., Ferrans, V. J., Jones, M., Cabin, H. S. and Roberts, W. C.: Calcific deposits developing in a bovine pericardial bioprosthetic valve 3 days after implantation. Circulation 63: 718-723, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03194-01 PA

PERIOD COVERED

October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Pulmonary Langerhans Cells in Patients With Fibrotic Lung Disorders

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

PI:	Victor J. Ferrans, Chief, Ultrastructure Section	PA	NHLBI
Others:	Oichi Kawanami, Visiting Expert, Ultrastructure Section	PA	NHLBI
	Francoise Basset, INSERM, Paris, France		
	Paul Soler, INSERM, Paris, France		
	Ronald G. Crystal, Chief, Pulmonary Branch	PB	NHLBI

COOPERATING UNITS (if any)

Pulmonary Branch, NHLBI
INSERM, Paris, France

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.2	PROFESSIONAL: 0.2	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)

Langerhans cells were found in lung biopsies in 1 of 9 control patients and in 20 of 160 patients with fibrotic lung disorders. Langerhans cells in lungs of these patients differed in morphology and in topographic distribution from the histiocytosis X cells in lungs of patients with pulmonary histiocytosis X.

PHS-6040
(Rev. 2-81)

Project Description: Langerhans' cells were found in lung biopsies in one of nine control patients and in 20 of 160 patients with fibrotic lung disorders, including 13 of 56 patients with idiopathic pulmonary fibrosis, two of nine patients with collagen vascular diseases, two of seven patients with hypersensitivity pneumonitis, and each of three patients with end stage fibrosis of uncertain cause. Langerhans' cells were not found in any of the 41 patients with sarcoidosis, the 35 patients with interstitial lung diseases associated with inhalation of inorganic dusts, the seven patients with pulmonary lymphangioleiomyomatosis, or the two patients with chronic eosinophilic pneumonia.

In the control patient, Langerhans' cells were found between epithelial cells in bronchioles. In patients with fibrotic lung disorders, Langerhans' cells were found in the epithelial layer of bronchioles and alveoli containing proliferating epithelial cells, i.e., either cuboidal epithelial cells of bronchiolar origin or type II alveolar epithelial cells. Severe fibrosis or squamous metaplasia were not prerequisites for the presence of Langerhans' cells. The motility of Langerhans' cells apparently was restricted, as they were not found in the air spaces in any of the biopsies, and they were not recovered from bronchoalveolar lavage fluid of any of the 97 patients studied, even though some of these patients had relatively numerous Langerhans' cells in lung biopsies. These observations are in sharp contrast to those in pulmonary histiocytosis X, in which histiocytosis X cells (HX cells) occur in granulomas, in alveolar interstitium, and between epithelial cells of the lower respiratory system. HX cells also migrate into air spaces, as shown by their occurrence in bronchoalveolar lavage fluid. The HX bodies in HX cells are morphologically similar to Langerhans' cell granules, but are more numerous and pleomorphic. HX cells are considered to be reactive or activated Langerhans' cells.

Publications: Kawanami O., Basset, F., Ferrans, V. J., Soler, P., and Crystal, R. G.: Pulmonary Langerhans' cells in patients with fibrotic lung disorders. Lab. Invest. 44: 227-233, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03195-01 PA

PERIOD COVERED
October 1, 1980, to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Myocardial Ultrastructure in the Cardiomyopathies

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

PI: Victor J. Ferrans, Chief, Ultrastructure Section PA NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.1	PROFESSIONAL: 0.1	OTHER:

CHECK APPROPRIATE BOX(ES)

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

☒ (a1) MINORS ☐ (a2) INTERVIEWS

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

A review is presented of the ultrastructural features of the heart in the major types of cardiomyopathies.

PHS-6040
(Rev. 2-81)

Project Description: A review is presented of the ultrastructural features of the heart in the major types of cardiomyopathies. The heart in hypertrophic cardiomyopathy shows disarray of muscle cells and of myofibrils; such changes can be found, albeit to a lesser extent, in other types of heart disease. In idiopathic cardiomyopathy of the congestive or ventricular dilated type, the heart shows hypertrophy, fibrosis and nonspecific degenerative alterations. Two types of cardiomyopathy, anthracycline-induced cardiomyopathy and the syndrome of infantile cardiomyopathy with histiocytoid change in the muscle cells, are described in detail. Only limited ultrastructural information is available on the endomyocardial diseases with and without eosinophilia. The infiltrative cardiomyopathies show a variety of changes, the nature of which depends on the type and localization (intracellular or interstitial) of the infiltrating material.

Publications: Ferrans, V. J.: Myocardial ultrastructure in the cardiomyopathies. In: Clinical and Experimental Aspects of Cardiomyopathy, M. Sekiguchi and E. J. G. Olsen (Eds.), University of Tokyo Press, 1980, pp. 107-139.

ANNUAL REPORT OF THE
LABORATORY OF BIOCHEMICAL GENETICS
NATIONAL HEART, LUNG AND BLOOD INSTITUTE
October 1, 1980 through September 30, 1981

Hybridomas were obtained that synthesize monoclonal antibodies directed against cells from the nervous system or synapse target cells. One antibody was found that binds to cell membrane molecules distributed in a large dorsal⟶ ventral topographic gradient in retina. The concentration of antigen detected was shown to be a function of the square of the circumferential distance from the ventral pole of the gradient towards the dorsal pole. Thus, the antigen defines a bilaterally symmetrical dorsal⟶ ventral axis of the retina and can be used as a marker of cell position in the retina. Cells which were dissociated from dorsal, middle, or ventral retina and cultured separately, continue to synthesize the antigen and accumulate the same amount of antigen as cells from the corresponding region in the intact retina _in ovo_. The number of antigen molecules detected per retina cell after prolonged culture was related to the prior position of the cells in the intact retina. The antigen was detected on all cells examined in dorsal and middle retina, but dorsal retina cells had more antigen than cells from middle retina. Thus, the antigen is distributed on the basis of cell position rather than cell type. ^{35}S-Protein in retina membranes was solubilized and the antigen was fractionated by protein A-Sepharose and hybridoma antibody-Sepharose column chromatography. SDS-polyacrylamide gel electrophoresis revealed one major band of ^{35}S-protein, approximately 60,000 M_r, and several minor ^{35}S-components. Further work is needed to determine whether the antigen functions as a specifier of positional information in the retina and to define the mechanism of establishing and perpetuating the gradient.

Stimulus-secretion coupling and signal transduction across cell membranes have been reported to be dependent on successive transmethylation reactions which convert phosphatidylethanolamine to phosphatidylcholine. Adenosine and S-adenosylhomocysteine analogs which have been reported to inhibit these transmethylation reactions were tested for their effects on reactions required for transsynaptic communication between neuroblastoma hybrid cells and myotubes. Inhibition of the transmethylation pathway for phosphatidylcholine synthesis by 3-deazaadensine or adenosine had no effect on $^{45}Ca^{2+}$ uptake by hybrid cells mediated by voltage-sensitive Ca^{2+} channels, on acetylcholine secretion at synapses, or on signal transduction mediated by nicotinic acetylcholine receptors; however, these processes were profoundly inhibited by 5'-deoxy-5'-isobutylthio-3-deazaadenosine (deaza-SIBA) (IC_{50} = 60 μM) or 5'-deoxy-5'-isobutylthioadenosine (SIBA) (IC_{50} = 140 μM). Deaza-SIBA or SIBA did not affect the transmethylation pathway for phosphatidylcholine synthesis in NG108-15 cells, but, instead, were shown to inhibit the synthesis of CDP-choline catalyzed by cholinephosphate cytidylyltransferase and thus, indirectly inhibit the CDP-choline pathway for phosphatidylcholine synthesis. These results show that the stimulus-secretion coupling and signal transduction reactions studied are not dependent on phospholipid methylation and suggest that they are functionally coupled to the rate of phosphatidylcholine synthesis via the CDP-choline pathway. The following mechanism was proposed which couples the rates of CDP-choline and phosphatidylcholine synthesis with the activity of action potential Ca^{2+} channels, neurotransmitter secretion, and receptor mediated signal transduction. Inhibition of CDP-choline synthesis by deaza-SIBA results in a decrease in the rate of synthesis of phosphatidylcholine and thus shifts the direction of the reaction catalyzed by choline phosphotransferase

from synthesis, to catabolism of phosphatidylcholine, thereby increasing cellular 1,2-diacyl-sn-glycerol and free fatty acids released by further catabolism of diacylglycerol. The changes in membrane structure due to breakdown of phosphatidylcholine in membranes and to increases in diacylglycerol and free fatty acid concentrations would be expected to affect many membrane functions.

Fusion of mouse spleen cells with P3X63 Ag8 mouse myeloma cells yielded 5 hybridoma cell lines that synthesize antibodies directed against antigens that were detected only in retina; antibodies were found that are specific for photoreceptor neurons, retina ganglion neurons, or neurons from the inner nuclear layer (horizontal, bipolar and amacrine neurons); other antibodies were restricted to the inner or outer synaptic layer of the retina. Thirteen antibodies bind to antigens distributed in synapse-like clusters in the synaptic layers of the retina. A search for monoclonal antibodies specific for molecules associated with synapses of striated muscle cells yielded 4 antibodies directed against clusters of antigen which localize at synaptic end-plates which copatch with nicotinic acetylcholine receptors. The antigen distributions are similar, but not identical to that of nicotinic acetylcholine receptors.

Neurons that innervate striated muscle cells can alter the expression of certain genes by muscle cells and determine the type of striated muscle cell that will develop; i.e., slow, fast red, or fast white muscle cells. Eleven monoclonal antibodies were found with muscle type specificity. A simple, reliable, rapid method was devised using monoclonal antibodies for the identification of striated muscle types, an assay often needed for the diagnosis of neuromuscular disorders in man, as well as for research on neuron-dependent regulation of gene expression by cultured muscle cells. Other hybridoma cell lines synthesize monoclonal antibodies specific for peripheral nerve cells, for certain cells in spinal cord or cerebral cortex, kidney glomeruli, or arteries and veins. Five antibodies also were obtained that bind preferentially to intercalated discs of cardiac muscle cells.

Fifty hybridoma cell lines were obtained that synthesize antibodies directed against molecules in rat cerebral cortex synaptosomal membrane preparations. Some of the antibodies bind to antigens in synaptosome membanes that were not detected in other tissues tested. Two antibodies were found with regional specificity within the nervous system; the antibodies bind to molecules in membranes from cerebral cortex and cerebellum that were not detected in retina. Two antibodies were found that affect synaptic transmission presynaptically by increasing $^{45}Ca^{2+}$ uptake and acetylcholine secretion from neural cells at synapses. Postsynaptic effects of antibodies such as myotube hyperpolarization or depolarization also were found. In addition, an antibody was found which binds to lipomodulin, a protein inhibitor of phospholipase A_2, thereby increasing the activity of the enzyme.

Various lines of neuroblastoma hybrid cells which form abundant synapses with cultured striated muscle cells possess both large, dense-core vesicles and small, clear vesicles, and secrete into the medium acetylcholine and a protein that induces the aggregation of nicotinic acetylcholine receptors on myotube plasma membranes. Cells from 3 lines of neuroblastoma x glioma hybrids were shown to have clonally inherited defects in synapse formation which were shown to be due to the absence of large dense-core vesicles and functional acetylcholine receptor aggregation protein. Cells from 2 lines with different types of synapse defects (141-3 cells synthesize and secrete acetylcholine but lack functional acetylcholine receptor aggregation protein, and N18TG-2 cells

synthesize little acetylcholine, but secrete acetylcholine receptor aggregation protein into the medium) were cocultured with muscle cells. Increases were found in the number of synapses formed by 141-3 cells and in the frequency of miniature end-plate potentials of myotubes. These results show in a model system that cooperative interactions between 3 cells can result in the formation of synapses and suggest that the acetylcholine receptor aggregation protein specifies the location of synapses mediated by nicotinic acetylcholine receptors.

Electron microscopy revealed regions of cultured myotubes with high acetylcholine receptor concentration were preferentially associated with: 1) dense, filamentous material attached to the cytoplasmic surface of the plasma membrane, 2) accumulations of microtubules, and 3) basal lamina. The synaptic basal lamina has been shown to contain unidentified molecules which induced aggregation of nicotinic acetylcholine receptors at former synaptic sites. A purified basement membrane glycoprotein, laminin, increased slighlty the number of acetylcholine receptor aggregates per myotube and also markedly enhanced the activity of the acetylcholine receptor aggregation protein synthesized by NG108-15 cells. These results suggest that laminin plays a role in the localization of nicotinic acetylcholine receptors at synaptic sites.

A soluble factor from bovine brain was found that is required for neurite extension by cells dissociated from 7-day chick embryo cerebral cortex that were cultured without serum. The factor was purified almost to homogeneity and was shown to be an acidic protein with a native M_r of approximately 75,000. Reduction with 2-mercaptoethanol converts the protein to subunits of approximately 37,000 M_r. Purified neurite extension protein induces neurite outgrowth at a concentration of 100 ng/ml of medium; i.e., approximately 1.2 nM. These results suggest that the neurite extension protein may be required for the assembly of neural circuits in the cerebral cortex.

Two types of voltage-sensitive Ca^{2+} channels were detected in neuroblastoma cells. One type of channel mediates Ca^{2+} action potentials and is activated by depolarization of the cell membrane potential to -50 mV; the second species of Ca^{2+} channel is activated at a more depolarized membrane potential (approximately -10 mV) and remains active for a relatively long time. The time constants for inactivation of the first and second species of Ca^{2+} channel are 20 and 2,000 msec, respectively. Evidence was obtained that suggests that long-lived activation of Ca^{2+} channels in neuroblastoma cells controls the activity of Ca^{2+} dependent K^+ channels and thereby regulates the membrane potential of the cells. In other systems Ca^{2+} channels with a long-lived activated state have been implicated in oscillatory membrane phenomena and in regulation of transmitter release at synapses.

The biosynthesis of the pentapeptides methionine-enkephalin and leucine-enkephalin which are endogenous ligands for opiate receptors was studied. Messenger RNA was isolated from bovine adrenal medulla and used as a template for the cell-free synthesis of enkephalin precursor protein. Incubation of the protein synthesized in the cell-free system with trypsin and carboxypeptidase-B liberated Met-enkephalin which was purified by immunoprecipitation and high-performance liquid chromatography. These results suggest that Met-enkephalin sequences in the precursor protein are separated from neighboring sequences by basic amino acid residues. Two fractions of polyadenylated mRNA coding for Met-enkephalin precursor protein were detected with chain lengths of 4,750 $\pm$ 450 nucleotides and 3,050 $\pm$ 550 base residues. The M_r of Met-enkephalin precursor protein was approximately 30,000-32,000

which accounts for only 18 to 28 of the bases in the mRNA molecules. The largest putative precursor of enkephalin that was detected in adrenal medulla extracts was 38,000 M_r. A nucleic acid probe for enkephalin mRNA is being used to assay enkephalin mRNA, to define the products of the enkephalin gene, and to explore the regulation of enkephalin gene expression.

In the course of studies on the metabolism of thyrotropin releasing hormone, we discovered a metabolite which we have referred to as histidyl-proline diketopiperazine. Over the past six years, evidence was obtained that suggests that the peptide is a neuroactive substance. The peptide has antidepressant activity, and produces hypothermia and a transient elevation in cyclic GMP levels when injected into the brain of rats. Dopamine uptake into synaptosomes is inhibited by histidyl-proline diketopiperazine; probably due to inhibition of sodium-potassium ATPase by the peptide. Others have shown that histidyl-proline diketopiperazine inhibits the release of prolactin in the pituitary.

While the studies alluded to above indicate that histidyl-proline diketopiperazine shows biological activity, they do not establish the peptide as a naturally occuring compound. The studies this year have been focussed in that direction. A specific antiserum was obtained from rabbits that recognizes histidyl-proline diketopiperazine, but not other peptides tested, which was used to isolate histidyl-proline diketopiperazine from brain. The amount of peptide detected in rat brain was 275-565 pmol/brain, approximately 2.5 times the concentration found for thyrotropin releasing hormone. Most of the cyclic dipeptide in brain is bound to high molecular weight material in the range of 70,000 M_r. Thyrotropin releasing hormone, but not histidyl-proline diketopiperazine was localized in synaptosomes. Both thyrotropin releasing hormone and histidyl-proline diketopiperazine were found in highest concentrations in pituitary and hypothalamus, but were found in other areas of brain as well. These data show that histidyl-proline diketopiperazine is a naturally occuring peptide.

A major focus of our attention during the past ten years has been to elucidate the mechanism by which the cellular concentration of cyclic AMP in *E. coli* is regulated. It has become clear that the transport of a variety of sugars into the cells is correlated with a lowering of cyclic AMP levels by way of an inhibition of the activity of adenylate cyclase. Two classes of sugar transport systems have been studied. The first, exemplified by glucose, transports sugars by a mechanism coupled to a transmembrane phosphorylation of the sugar. We have found that the activity of adenylate cyclase is regulated by the degree of phosphorylation of some as yet unidentified factor in this transport system. The second transport mechanism, exemplified by lactose, catalyzes the transport of sugars across the cell membrane by a proton symport and depends on membrane potential as a driving force. We have found that the membrane potential regulates the activity of adenylate cyclase in some way that is not clearly understood. In both of these regulatory mechanisms, the interaction of cells with sugar substrates catalyzes the transport of sugars coupled to an *inhibition* of adenylate cyclase activity. We have now found that some *E. coli* strains carrying mutations in the permease for lactose show an effect opposite to that of the wild-type strain; the accumulation of cyclic AMP by intact cells is *stimulated* by lactose, but only when the mutant permease is present. Furthermore, we have found that insertion of lactose permease into the membrane of cells can produce a change in the specific activity of adenylate cyclase; induction of the wild-type transporter is associated with a decrease in the specific activity, while implantation of a mutant form of lactose permease

can lead to an increase in the specific activity. From these studies, it appears that the condition of the lactose transporter in the cell membrane influences the activity of adenylate cyclase.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00009-07 LBG

PERIOD COVERED
October 1, 1980 - September 30, 1981

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

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COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemical Genetics

SECTION

Section of Molecular Biology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
12	10	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)

A retina cell membrane protein that is distributed in a dorsal ⟶ ventral topographic gradient in retina was purified 3000-fold. We have tested the hypothesis that signal transduction through cell membranes is dependent on phosphatidylcholine synthesis by transmethylation and find that stimulus-secretion coupling and signal transduction through cell membranes at synapses are profoundly inhibited by inhibition of the CDP-choline pathway for phosphatidylcholine synthesis but not the transmethylation pathway. Monoclonal antibodies were obtained which are specific for the inner or outer synaptic layer of the retina, for certain types of neurons in retina, for antigens localized in clusters at synaptic end-plates of striated muscle cells and for different types of striated muscle cells. Two antibodies increase $^{45}Ca^{2+}$ uptake and acetylcholine secretion by neuroblastoma hybrid cells. Another antibody binds to lipomodulin, a protein inhibitor of phospholipase A_2. Cells with a clonally inherited defect in synapse formation were shown to be deficient in nicotinic acetylcholine receptor aggregation protein and large dense-core vesicles. The defect was partially repaired by the addition of the receptor aggregation protein to the medium. A protein from bovine brain was purified almost to homogeneity which induces neurite extension by chick embryo cerebral cortex neurons at nM concentrations. Neurons were isolated from chick or rat embryo spinal cord which synthesize acetylcholine and survive only in the presence of muscle cells or a factor secreted by muscle cells into the medium.

PHS-6040
(Rev. 2-81)

Project Description:

Major Findings:

Hybridomas were obtained that synthesize monoclonal antibodies directed against cells from the nervous system or synapse target cells. One antibody was found that binds to cell membrane molecules distributed in a large dorsal⟶ ventral topographic gradient in retina. The concentration of antigen detected was shown to be a function of the square of the circumferential distance from the ventral pole of the gradient towards the dorsal pole. Thus, the antigen defines a bilaterally symmetrical dorsal⟶ ventral axis of the retina and can be used as a marker of cell position in the retina. Cells which were dissociated from dorsal, middle, or ventral retina and cultured separately, continue to synthesize the antigen and accumulate the same amount of antigen as cells from the corresponding region in the intact retina in ovo. The number of antigen molecules detected per retina cell after prolonged culture was related to the prior position of the cells in the intact retina. The antigen was detected on all cells examined in dorsal and middle retina, but dorsal retina cells had more antigen than cells from middle retina. Thus, the antigen is distributed on the basis of cell position rather than cell type. ^{35}S-Protein in retina membranes was solubilized and the antigen was fractionated by protein A-Sepharose and hybridoma antibody-Sepharose column chromatography. SDS-polyacrylamide gel electrophoresis revealed one major band of ^{35}S-protein, approximately 60,000 M_r, and several minor ^{35}S-components. Further work is needed to determine whether the antigen functions as a specifier of positional information in the retina and to define the mechanism of establishing and perpetuating the gradient.

Stimulus-secretion coupling and signal transduction across cell membranes have been reported to be dependent on successive transmethylation reactions which convert phosphatidylethanolamine to phosphatidylcholine. Adenosine and S-adenosylhomocysteine analogs which have been reported to inhibit these transmethylation reactions were tested for their effects on reactions required for transsynaptic communication between neuroblastoma hybrid cells and myotubes. Inhibition of the transmethylation pathway for phosphatidylcholine synthesis by 3-deazaadensine or adenosine had no effect on $^{45}Ca^{2+}$ uptake by hybrid cells mediated by voltage-sensitive Ca^{2+} channels, on acetylcholine secretion at synapses, or on signal transduction mediated by nicotinic acetylcholine receptors; however, these processes were profoundly inhibited by 5'-deoxy-5'-isobutylthio-3-deazaadenosine (deaza-SIBA) (IC_{50} = 60 μM) or 5'-deoxy-5'-isobutylthioadenosine (SIBA) (IC_{50} = 140 μM). Deaza-SIBA or SIBA did not affect the transmethylation pathway for phosphatidylcholine synthesis in NG108-15 cells, but, instead, were shown to inhibit the synthesis of CDP-choline catalyzed by cholinephosphate cytidylyltransferase and thus, indirectly inhibit the CDP-choline pathway for phosphatidylcholine synthesis. These results show that the stimulus-secretion coupling and signal transduction reactions studied are not dependent on phospholipid methylation and suggest that they are functionally coupled to the rate of phosphatidylcholine synthesis via the CDP-choline pathway. The following mechanism was proposed which couples the rates of CDP-choline and phosphatidylcholine synthesis with the activity of action potential Ca^{2+} channels, neurotransmitter secretion, and receptor mediated signal transduction. Inhibition of CDP-choline synthesis by deaza-SIBA results in a decrease in the rate of synthesis of phosphatidylcholine and thus

shifts the direction of the reaction catalyzed by choline phosphotransferase from synthesis, to catabolism of phosphatidylcholine, thereby increasing cellular 1,2-diacyl-sn-glycerol and free fatty acids released by further catabolism of diacylglycerol. The changes in membrane structure due to breakdown of phosphatidylcholine in membranes and to increases in diacylglycerol and free fatty acid concentrations would be expected to affect many membrane functions.

Fusion of mouse spleen cells with P3X63 Ag8 mouse myeloma cells yielded 5 hybridoma cell lines that synthesize antibodies directed against antigens that were detected only in retina; antibodies were found that are specific for photoreceptor neurons, retina ganglion neurons, or neurons from the inner nuclear layer (horizontal, bipolar and amacrine neurons); other antibodies were restricted to the inner or outer synaptic layer of the retina. Thirteen antibodies bind to antigens distributed in synapse-like clusters in the synaptic layers of the retina. A search for monoclonal antibodies specific for molecules associated with synapses of striated muscle cells yielded 4 antibodies directed against clusters of antigen which localize at synaptic end-plates which copatch with nicotinic acetylcholine receptors. The antigen distributions are similar, but not identical to that of nicotinic acetylcholine receptors.

Neurons that innervate striated muscle cells can alter the expression of certain genes by muscle cells and determine the type of striated muscle cell that will develop; i.e., slow, fast red, or fast white muscle cells. Eleven monoclonal antibodies were found with muscle type specificity. A simple, reliable, rapid method was devised using monoclonal antibodies for the identification of striated muscle types, an assay often needed for the diagnosis of neuromuscular disorders in man, as well as for research on neuron-dependent regulation of gene expression by cultured muscle cells. Other hybridoma cell lines synthesize monoclonal antibodies specific for peripheral nerve cells, for certain cells in spinal cord or cerebral cortex, kidney glomeruli, or arteries and veins. Five antibodies also were obtained that bind preferentially to intercalated discs of cardiac muscle cells.

Fifty hybridoma cell lines were obtained that synthesize antibodies directed against molecules in rat cerebral cortex synaptosomal membrane preparations. Some of the antibodies bind to antigens in synaptosome membanes that were not detected in other tissues tested. Two antibodies were found with regional specificity within the nervous system; the antibodies bind to molecules in membranes from cerebral cortex and cerebellum that were not detected in retina. Two antibodies were found that affect synaptic transmission presynaptically by increasing $^{45}Ca^{2+}$ uptake and acetylcholine secretion from neural cells at synapses. Postsynaptic effects of antibodies such as myotube hyperpolarization or depolarization also were found. In addition, an antibody was found which binds to lipomodulin, a protein inhibitor of phospholipase A_2, thereby increasing the activity of the enzyme.

Various lines of neuroblastoma hybrid cells which form abundant synapses with cultured striated muscle cells possess both large, dense-core vesicles and small, clear vesicles, and secrete into the medium acetylcholine and a protein that induces the aggregation of nicotinic acetylcholine receptors on myotube plasma membranes. Cells from 3 lines of neuroblastoma x glioma hybrids were shown to have clonally inherited defects in synapse formation which were shown to be due to the absence of large dense-core vesicles and functional

acetylcholine receptor aggregation protein. Cells from 2 lines with different types of synapse defects (141-3 cells synthesize and secrete acetylcholine but lack functional acetylcholine receptor aggregation protein, and N18TG-2 cells synthesize little acetylcholine, but secrete acetylcholine receptor aggregation protein into the medium) were cocultured with muscle cells. Increases were found in the number of synapses formed by 141-3 cells and in the frequency of miniature end-plate potentials of myotubes. These results show that cooperative interactions between 3 cells can result in the formation of synapses and suggest that the acetylcholine receptor aggregation protein functions as a specifier of position for synapses mediated by nicotinic acetylcholine receptors.

A soluble factor from bovine brain was found that is required for neurite extension by cells dissociated from 7-day chick embryo cerebral cortex that were cultured without serum. The factor was purified almost to homogeneity and was shown to be an acidic protein with a native M_r of approximately 75,000. Reduction with 2-mercaptoethanol converts the protein to subunits of approximately 37,000 M_r. Purified neurite extension protein induces neurite outgrowth at a concentration of 100 ng/ml of medium; i.e., approximately 1.2 nM. These results suggest that the neurite extension protein may be required for the assembly of neural circuits in the cerebral cortex.

Two types of voltage-sensitive Ca^{2+} channels were detected in neuroblastoma cells. One type of channel mediates Ca^{2+} action potentials and is activated by depolarization of the cell membrane potential to -50 mV; the second species of Ca^{2+} channel is activated at a more depolarized membrane potential (approximately -10 mV) and remains active for a relatively long time. The time constants for inactivation of the first and second species of Ca^{2+} channel are 20 and 2,000 msec, respectively. Evidence was obtained that suggests that long-lived activation of Ca^{2+} channels in neuroblastoma cells controls the activity of Ca^{2+} dependent K^+ channels and thereby regulates the membrane potential of the cells. In other systems Ca^{2+} channels with a long-lived activated state have been implicated in oscillatory membrane phenomena and in regulation of transmitter release at synapses.

Significance to Biomedical Research:

A protein was isolated from plasma membranes of retina cells which is distributed in a bilaterally symmetrical dorsal⟶ ventral topographic gradient in the retina and which defines an axis of the retina. The spatial organization of the molecules in retina suggest that they may function as cell determinants of positional information.

Stimulus secretion coupling and signal transduction through cell membranes were found to be functionally coupled to the rate of phosphatidylcholine synthesis via the CDP-choline pathway but not via the transmethylation pathway for phosphatidylcholine synthesis.

Monoclonal antibodies were obtained with specificity for molecules localized at synaptic sites, or for certain types of neurons, and for types of striated muscle cells. Other antibodies were found that affect synaptic functions.

Proposed Course:

Further work is needed to purify and characterize certain antigens and to define the functions of the antigens.

Publications:

1. Trisler, G.D., Schneider, M.D. and Nirenberg, M.: A topographic gradient of molecules in retina can be used to identify neuron position. Proc. Natl. Acad. Sci., USA, 78: 2145-2149 (1981).

2. Hirata, F., Del Carmine, R., Nelson, C.A., Axelrod, J., Schiffmann, E., Warabi, A., De Blas, A.L., Nirenberg, M., Manganiello, R., Vaughan, M., Kumagai, S., Green, I., Decker, J. L. and Steinberg, A.D.: Presence of autoantibody for phospholipase inhibitory protein, lipomodulin, in patients with rheumatic diseases. Proc. Natl. Acad. Sci., USA, 78: 3190-3194, (1981).

3. De Blas, A. L., Busis, N. A. and Nirenberg, M.: Monoclonal antibodies to synaptosomal membrane molecules. In: Monoclonal Antibodies Against Neuronal Antigens, ed. by McKay, R., Raff, M. and Reichardt, L., Cold Spring Harbor Monograph. In Press (1981).

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10. Spector, Ilan: Electrophysiology of Clonal Nerve Cell Lines. In: Excitable Cells in Tissue Culture, pp. 247-277, Ed. by P. G. Nelson and M. Lieberman. Plenum Pub. Corp. (1981).

11. Spector, I., Fishman, M. and Dragsten, P.: Immobilization of Concanavalin A receptors during differentiation of neuroblastoma cells. Nature, 290: 781-783 (1981).

12. Fishman, M. C. and Spector, I.: Potassium current suppression by quinidine reveals additional calcium currents in neuroblastoma cells. Proc. Natl. Acad. Sci., USA. In Press (1981).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00017-06 LBG

PERIOD COVERED
October 1, 1980 - September 30, 1981

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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	Hans Bauer	Visiting Scientist	LDN NICHD
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COOPERATING UNITS (if any)

Laboratory of Developmental Neurobiology, NICHD
Laboratory of Developmental Biology & Anomalies, NIDR

LAB/BRANCH
Laboratory of Biochemical Genetics

SECTION
Section on Molecular Biology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.0	4.0	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)

Our aim has been to reveal the topographic distribution of neurotransmitter receptors on nerve and muscle cells, while relating this distribution to the development and function of synapses. To this purpose, we have used α-bungarotoxin as a specific probe for the visualization and quantitation of nicotinic acetylcholine receptor sites. Our recent work has emphasized a model system for studying the neural control of nicotinic acetylcholine receptor distribution on skeletal muscle fibers. This system is based on the finding that embryonic and clonal nerve cells produce a factor which induces aggregation of receptors on cultured skeletal myotubes. We have now provided electron microscopic evidence for the involvement of cytoskeletal structures and extracellular matrix structures in the formation and maintenance of receptor aggregates. We have also provided morphological and kinetic evidence that laminin, a basement membrane glycoprotein, may be involved in the induction of receptor aggregates by neuronal factors.

PHS-6040
(Rev. 2-81)

Project Description:

Objectives:

Our aim has been to reveal the topographic distribution of neurotransmitter receptors on nerve and muscle cells, while relating this distribution to the development and function of synapses. Our recent work has focussed on the extrinsic and intrinsic influences on the distribution of nicotinic acetylcholine receptors (AChR) on the surface of the developing muscle fiber. In particular, we have been investigating the mechanisms whereby soluble, macromolecular factors from neurons can induce localized aggregation of AChR as occurs in the developing neuromuscular synapse.

Methods:

We have stained skeletal myotubes grown in monolayer culture with rhodamine-labeled α-bungarotoxin (αBT) in order to visualize AChR sites with the fluorescence microscope. The distribution of basement membrane proteins in the same cultures, and in sections of muscle tissue, was determined by indirect immunofluorescence, using antisera against purified proteins.

Alpha-bungarotoxin coupled to horseradish peroxidase was used to examine the distribution of AChR on myotube surfaces at the electron microscopic level.

AChR aggregating material was prepared from culture medium conditioned by clonal nerve cell lines.

Major Findings:

On electron microscopic examination, regions of cultured myotubes with high AChR density (AChR aggregates) showed a preferential association with: 1) dense, filamentous material attached to the cytoplasmic surface of the plasma membrane; 2) accumulations of microtubules; 3) basal lamina and other extracellular matrix structures.

Fluorescence double-staining for AChR and basement membrane proteins revealed the preferential association of laminin (the major basement membrane glycoprotein) and heparan sulfate proteoglycan with aggregated AChR on the cultured myotube surface. Addition of laminin to myotube cultures caused a small increase in the number of AChR aggregates. Furthermore, low concentrations of laminin markedly enhanced the receptor aggregating activity of neuronal conditioned medium.

These results provide evidence for a role of one or more basement membrane proteins in the postsynaptic organization of AChR.

Significance to Biomedical Research:

Knowledge of the topographical distribution of neurotransmitter receptors on nerve and muscle cells 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. Our studies on the AChR aggregation factor may lead to a better understanding of the mechanisms whereby neurons control or modulate the distribution of receptors on muscle fibers and on other neurons, during synapse development and after.

Proposed Course:

1) We plan to continue the detailed light- and electronmicroscopic analysis of AChR aggregate formation and the underlying structural mechanisms, using recently developed morphological techniques.

2) The possible interactions between basement membrane proteins, AChR, and the neuronal AChR aggregating factor will be examined to clarify the role of these proteins in AChR organization. The mechanism by which laminin enhances the neuronal factor-induced AChR aggregation will be examined in kinetic and pharmacological studies.

Publications:

1) Daniels, M.P. and Vogel, Z.: Localization of α-bungarotoxin binding sites in synapses of the developing chick retina. Brain Res., 201, 45-56 (1980).

2) Christian, C.N., Bergey, G.K., Daniels, M.P. and Nelson, P.G.: Cell interactions in nerve and muscle cell cultures. J. Exp. Biol., 89, 85-101 (1980).

3) Bauer, H.C., Daniels, M.P., Pudimat, P.A., Jacques, L., Sugiyama, H. and Christian, C.N.: Characterization and partial purificiation of a neuronal factor which increases acetylcholine receptor aggregation on cultured muscle cells. Brain Res., 209, 395-404 (1981).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00018-04 LBG

PERIOD COVERED
October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)

Regulation of the biosynthesis and secretion of opioid peptides

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

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OTHER:	Satyaprabha Dandekar	Visiting Fellow	LBG, NHLBI
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COOPERATING UNITS (if any)

None

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SECTION
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INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.8	2.5	0.3 (Summer Student)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The biosynthesis of methionine-enkephalin and leucine-enkephalin, pentapeptides which are endogenous ligands for the opiate receptor, is under investigation. Messenger RNA obtained from bovine adrenal medulla, a rich source of enkephalins, has been isolated and used as a template for the synthesis of the primary enkephalin gene product in cell-free translation systems. The synthesis of an enkephalin-containing putative precursor protein was demonstrated by the liberation of enkephalin sequences from protein by specific proteolysis, followed by immunoprecipitation and high-performance liquid chromatography. The molecular weight of the enkephalin-containing protein is probably 30-32,000, while the messenger RNA coding for this protein is heterogeneous and consists of possibly two fractions of apparent size 4750 and 3000 nucleotides. Monoclonal antibodies and conventional antibodies to enkephalin precursor protein are being prepared, and a nucleic acid probe for enkephalin messenger RNA is being utilized to identify and quantitate enkephalin messenger RNA. Using the methodology above, regulation of enkephalin precursor gene expression is under investigation.

PHS-6040
(Rev. 2-81)

Objective:

The pentapeptides methionine-enkephalin (Tyr-Gly-Gly-Phe-Met) and leucine-enkephalin (Tyr-Gly-Gly-Phe-Leu) are endogenous opiate-receptor ligands that may function as neurotransmitters or neuromodulators. Recent studies by other laboratories on enkephalin biosynthesis have led to the characterization of a class of peptides, isolated from bovine adrenal medulla and guinea pig striatum, that contain multiple enkephalin sequences which are generally separated from neighboring sequences by pairs of basic amino acid residues that probably function as signals for processing. As an alternative approach, we have initiated a characterization of the primary enkephalin gene product(s) synthesized in cell-free translation systems. Our objectives are: (1) to determine the size of the enkephalin gene product synthesized in the absence of post-translational proteolytic processing or glycosylation; (2) to determine the size of enkephalin precursor mRNA; and (3) to develop techniques to assay the synthesis of both enkephalin precursor protein and enkephalin mRNA in order to study enkephalin gene organization and expression.

Methods Employed: Discussed in Major Findings below.

Major Findings:

Adrenal medullary mRNA (polyadenylated RNA), when translated in the rabbit reticulocyte and wheat germ cell-free translation systems, directs the incorporation of [^{35}S]methionine into many proteins, as analyzed by polyacrylamide gel electrophoresis. None of these synthesized proteins appears to be specifically recognized by enkephalin antisera, but after these proteins are treated with trypsin and carboxypeptidase B, [^{35}S]Met-enkephalin (0.2 fmol/μg mRNA) can be isolated by immunoprecipitation with anti-Met-enkephalin sera, and identified by high-performance liquid chromatographic retention time and immunological specificity. Both trypsin and carboxypeptidase B are required to generate [^{35}S]Met-enkephalin from translation products; thus, the Met-enkephalin sequences are probably separated from neighboring sequences by basic amino acid residues.

The size of the cell-free translated Met-enkephalin-containing protein was examined by SDS-polyacrylamide gel electrophoresis followed by proteolytic digestion and immunoprecipitation. A protein of M_r 30-32,000 was consistently found; this molecular weight is slightly smaller than that of the largest enkephalin putative precursor extracted from adrenal medulla (M_r 38,000 by gel filtration in our laboratory). The difference may be due to glycosylation of the precursor after translation. Whether the 30-32,000 protein represents the full primary enkephalin gene product still remains to be unequivocally established. In order to quantitate enkephalin precursor protein more directly, antibodies (monoclonal and conventional) are being prepared against a purified intermediate fragment of the enkephalin precursor, and against the peptide Met-enkephalin[Arg^{6}Phe^7], which may be at the carboxyl terminal end of the precursor.

Polyadenylated mRNA coding for Met-enkephalin-containing protein was found to be heterogeneous by agarose gel electrophoresis, as determined by translation assays. In the presence of the denaturant methylmercuric hydroxide, two major fractions were obtained having chain lengths of 4750 $\pm$ 450 nucleotides and 3050 $\pm$ 550 nucleotides. In the absence of denaturant, two major fractions were

electrophoretically resolved, having apparent sizes of 30S and 22S, or 5750 ± 450 and 2850 ± 250 nucleotides, respectively. Messengers of these sizes contain information to code for proteins much larger than 30,000. The discrepancy between the size of the protein synthesized and the information content of its mRNA is currently under investigation. The technique of gel-blot hybridization (Northern blotting) has been set up in order to use a custom-synthesized dodecadeoxynucleotide probe that will specifically hybridize with the nucleotide sequence of the mRNA coding for the Met-enkephalin amino acid sequence. Preliminary results confirm the molecular weight values obtained by the translation method.

Significance to Biomedical Research:

Enkephalinergic neurons constitute probably a very important and extensive system of the nervous system that may be involved in pain perception, stress, and behavior. Enkephalin-secreting glandular cells, such as adrenal chromaffin cells, may be likewise important. The values determined for the size of the primary enkephalin gene product and its mRNA(s) are essential structural information for the study of enkephalin gene organization through cloning and other techniques. Almost nothing is currently known about regulation of the biosynthesis of enkephalins. The progress described above in the development of methodology to assay the synthesis of enkephalin precursor mRNA and protein is crucial to the future study of this regulation under normal physiological conditions and in pathological conditions such as opiate dependence.

Future Course:

1. Synthesis of cDNA complementary to enkephalin mRNA using the synthetic dodecadeoxynucleotide described above as a primer for reverse transcriptase, then sequencing the cDNA.

2. Cloning of cDNA complementary to enkephalin precursor mRNA in an *E. coli* plasmid or phage system. Clones may be used as specific gene probes and may enable the complete sequencing of enkephalin precursor mRNA.

3. Preparation and characterization of antisera and monoclonal antibodies against enkephalin precursor protein to allow direct immunoprecipitation of this protein.

4. Generalization of cell-free translation studies and mRNA size analysis to include enkephalin mRNA from other tissues, such as striatum.

5. Studies on the regulation of enkephalin transcription and translation using cultured cell systems.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00151-11 LBG

PERIOD COVERED
October 1, 1980 - September 30, 1981

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

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COOPERATING UNITS (if any)

None

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INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.5	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)

Our continuing interest in this area has been to develop an understanding of the role and mechanism of regulation of the cyclic nucleotides cyclic AMP and cyclic GMP in E. coli. Our emphasis this year has been on the mechanism by which the sugar lactose regulates the activity of adenylate cyclase. In wild-type strains of E. coli, the transport of lactose is correlated with an inhibition of adenylate cyclase activity. The mechanism of this effect is complex, involving regulation by mechanisms involving phosphorylation as well as membrane potential. In some E. coli strains harboring mutant forms of lactose permease, exposure of the cells to lactose produces a stimulation of adenylate cyclase. These findings suggest an additional level of complexity of regulation of adenylate cyclase activity.

PHS-6040
(Rev. 2-81)

PROJECT DESCRIPTION:

Major Findings:

A major focus of our attention during the past ten years has been to elucidate the mechanism by which the cellular concentration of cyclic AMP in E. coli is regulated. It has become clear that the transport of a variety of sugars into the cells is correlated with a lowering of cyclic AMP levels by way of an inhibition of the activity of adenylate cyclase. Two classes of sugar transport systems have been studied. The first, exemplified by glucose, transports sugars by a mechanism coupled to a transmembrane phosphorylation of the sugar. We have found that the activity of adenylate cyclase is regulated by the degree of phosphorylation of some as yet unidentified factor in this transport system. The second transport mechanism, exemplified by lactose, catalyzes the transport of sugars across the cell membrane by a proton symport and depends on membrane potential as a driving force. We have found that the membrane potential regulates the activity of adenylate cyclase in some way that is not clearly understood. In both of these regulatory mechanisms, the interaction of cells with sugar substrates catalyzes the transport of sugars coupled to an inhibition of adenylate cyclase activity. We have now found that some E. coli strains carrying mutations in the permease for lactose show an effect opposite to that of the wild-type strain; the accumulation of cyclic AMP by intact cells is stimulated by lactose, but only when the mutant permease is present. Furthermore, we have found that insertion of lactose permease into the membrane of cells can produce a change in the specific activity of adenylate cyclase; induction of the wild-type transporter is associated with a decrease in the specific activity, while implantation of a mutant form of lactose permease can lead to an increase in the specific activity. From these studies, it appears that the condition of the lactose transporter in the cell membrane influences the activity of adenylate cyclase.

Proposed Course of Research:

As a result of the more recent studies, the mechanism of regulation of adenylate cyclase in E. coli appears to be more complex than previously realized. Regulation involves phosphorylation at some level, membrane potential and also the structure of the membrane. Our original intention to more fully understand the mechanism of regulation of adenylate cyclase was to reconstitute the system from purified components. While this is still our long-term objective, the direction of our efforts will be to carry out reconstitution studies under conditions where factors such as phosphorylation, membrane potential and membrane structure can be controlled.

Publications:

1) Peterkofsky, A. and Gazdar, C.: Stimulation of Escherichia coli adenylate cyclase by lactose in strains carrying mutations in lactose permease. Bioscience Reports 1: 53-60, 1981.

2) Peterkofsky, A.: Escherichia coli adenylate cyclase as a sensor of sugar transport function. In: Dumont, J.E., Greengard, P. and Robison, G.A. (Eds.): Advances in Cyclic Nucleotide Research, Raven Press, New York, Vol. 14, pp. 215-228, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00152-07 LBG

PERIOD COVERED
October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)

Metabolism of Peptide Hormones

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

PI:	Alan Peterkofsky	Research Chemist Chief, Section on Macromolecules	LBG, NHLBI
OTHER:	Fiorenzo Battaini	Visiting Associate	LBG, NHLBI
	Yitzhak Koch	Visiting Associate	LBG, NHLBI
	Yoshiyuki Takahara	Guest Worker	LBG, NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemical Genetics

SECTION

Section on Macromolecules

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	3.5	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

We have continued our studies on the metabolite of thyrotropin releasing hormone known as histidyl-proline diketopiperazine. During the past year, we have developed an antiserum in rabbits that is highly specific for histidyl-proline diketopiperazine. This antiserum does not bind thyrotropin releasing hormone or other related peptides and can distinguish between the natural form of histidyl-proline diketopiperazine and a diastereoisomer. The antiserum was used as a tool for developing an isolation and assay procedure for histidyl-proline diketopiperazine from rat brain. It was found that the cyclic dipeptide occurs naturally in a bound form associated with some material of molecular weight approximately 70,000. A procedure was devised for releasing the low molecular weight peptide from the bound form. The concentration of histidyl-proline in rat brain was found to be approximately 2.5 times higher than that of thyrotropin releasing hormone. A regional distribution of the peptide indicated that it was found in highest concentrations in pituitary and hypothalamus.

PHS-6040
(Rev. 2-81)

Major Findings:

In the course of studies of the metabolism of thyrotropin releasing hormone, we discovered a metabolite which we have referred to as histidyl-proline diketopiperazine. Over the past six years, evidence has accumulated that provides a basis for the conclusion that the peptide is a neuroactive substance. When it is injected into the brain of rats, it shows antidepressant activity and also produces hypothermia. Furthermore, the peptide produces a transient elevation in cyclic GMP levels in brain. Dopamine uptake into synaptosomes is inhibited by histidyl-proline diketopiperazine; this action is probably explained by the action of the peptide as an inhibitor of sodium-potassium ATPase. At the pituitary level, other workers have shown that histidyl-proline diketopiperazine inhibits the release of prolactin.

While the studies alluded to above indicate that histidyl-proline diketopiperazine shows biological activity, they do not establish the peptide as a naturally occuring compound. The studies this year have been focussed in that direction. We have developed a specific antiserum in rabbits that recognizes histidyl-proline diketopiperazine but none of a variety of other peptides tested. This antiserum has been used as the basis for devising a procedure for isolating histidyl-proline diketopiperazine from brain in a form that can be assayed. Using this assay we found that the peptide was present in rat brain in the range of 275-565 pmol/brain, approximately 2.5 times the concentration determined for thyrotropin releasing hormone. The preponderance of the cyclic dipeptide is found in brain bound to high molecular weight material in the range of 70,000 M_r. A study of the subcellular distribution of histidyl-proline diketopiperazine and thyrotropin releasing hormone indicated that the releasing factor was localized in synaptosomes while the diketopiperazine was not. A determination of the regional distribution of thyrotropin releasing hormone and histidyl-proline diketopiperazine indicated that both peptides are found in highest concentrations in pituitary and hypothalamus, but are detectable in other areas of brain as well. These data allow us to conclude that histidyl-proline diketopiperazine is a naturally occuring peptide.

Proposed Course of Research:

Our finding that histidyl-proline diketopiperazine occurs naturally in brain in a bound form warrants further attention. It is our intention to attempt to purify the high molecular weight material and characterize it. The nature of the complex between histidyl-proline diketopiperazine should be studied. Furthermore, we would like to determine if the bound form of the peptide is biologically active.

Publications:

1) Yanagisawa, T., Prasad, C. and Peterkofsky, A.: The subcellular and organ distribution and natural form of histidyl-proline diketopiperazine in rat brain determined by a specific radioimmunoassay. J. Biol. Chem. 255: 10290-10298, 1980.

2) Peterkofsky, A. and Battaini, F.: The biological activities of the neuropeptide histidyl-proline diketopiperazine. Neuropeptides 1: 105-118, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00153-03 LBG

PERIOD COVERED
October 1, 1980 - September 30, 1981

TITLE OF PROJECT (80 characters or less)

Biochemical characterization of α-adrenergic receptors

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

PI:	Steven Sabol	Medical Officer	LBG, NHLBI
OTHER:	Daphne Atlas	Visiting Scientist	LBC, NIADDK

COOPERATING UNITS (if any)

Laboratory of Bio-organic Chemistry, NIADDK

LAB/BRANCH

Laboratory of Biochemical Genetics

SECTION

Section on Molecular Biology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.05	0.05	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 program was begun to devise novel molecular probes and affinity labels for α-adrenergic receptors. Several analogues of clonidine (an α_2-adrenergic receptor partial agonist, and other α-adrenergic ligands were synthesized and tested for binding to α-receptors of rat brain and NG108-15 neuroblastoma x glioma hybrid cells. In the latter system, α-receptor binding of $[^3H]$clonidine was partially characterized and correlated with α_2-receptor-mediated inhibition of adenylate cyclase. A hydroxyphenacetyl derivative of p-aminoclonidine was prepared as an α-receptor ligand that may be iodinated; this compound possesses both strong α-receptor partial agonist activity and moderate opiate-receptor agonist activity.

Work done on this project during the period covered by this report was limited to the publication of most of the research findings. This report is submitted to record this publication.

PHS-6040
(Rev. 2-81)

PROJECT DESCRIPTION: Z01 HL 00153-03 LBG

Objective:

Little is known about the molecular properties of α-receptors. Recent work has resulted in the differentiation of at least two classes of α-receptors, α_1 and α_2. Clonal NG108-15 neuroblastoma x glioma hybrid cells possess α_2 receptors that mediate the inhibition of adenylate cyclase. We previously proposed that activation of α_2-receptors, but not necessarily α_1-receptors, may be generally associated with the inhibition of adenylate cyclase. One approach to the biochemical characterization of α-receptors is to prepare suitable affinity labels that will specifically react covalently with either α_1 or α_2-receptors. In addition, there is a need for radioiodinated and fluorescent α-receptor probes.

Major Findings:

Clonidine, p-aminoclonidine, and N-(4-hydroxyphenacetyl-4-aminoclonidine (HP-aminoclonidine) were shown to be potent partial agonists in the α-adrenergic-receptor mediated inhibition on NG108-15 adenylate cyclase (K_{Dapp} 300, 50, 130 nM, respectively); these compounds also bind with high affinity to α_2-receptors of rat brain and NG108-15 membranes, as determined by displacement of bound [^{3}H]clonidine. For brain membranes, binding of [^{3}H]-clonidine that is displaceable by the α-antagonist phentolamine has been previously characterized as binding to α_2-receptors. We characterized [^{3}H]-clonidine binding to NG108-15 membranes in order to correlate binding affinities with inhibition of adenylate cyclase in the same system. [^{3}H]Clonidine binds to NG108-15 membranes with K_D values of 1.7 and 33 nM for apparent high- and low affinity sites, respectively. The relative proportions of these apparent classes of sites varied among different preparations. Classical α-adrenergic agonists and antagonists displace [^{3}]clonidine from these sites. p-Aminoclonidine and HP-aminoclonidine displace [^{3}H]clonidine from the high-affinity sites with K_D values of 2.3 and 5.8 nM, respectively. Similar affinities of the analogues for rat brain α_2-receptors were found. Radioiodination of HP-aminoclonidine was attempted, but so far without retention of α-adrenergic activity.

In the NG108-15 system, the affinities of ligands for the high-affinity α-receptor binding sites were found to be many times greater than the corresponding potencies in the inhibition of NG108-15 adenylate cyclase. This discrepancy can be explained by the findings that GTP, ATP, Na^+ ions, and possibly other components of the adenylate cyclase system inhibit binding of α-receptor agonists to the high affinity site. GTP and probably Na^+ ions are required for α-receptor-mediated inhibition of adenylate cyclase; thus, the high-affinity [^{3}H]clonidine site may represent uncoupled receptors, while the low affinity sites may represent receptors coupled to adenylate cyclase.

Significance to Biomedical Research:

Alpha-adrenergic receptors mediate responses to norepinephrine and epinephrine in the autonomic nervous system and in a variety of non-neuronal cells. The use of affinity labels, particularly those preferring α_1 or α_2-receptors, should enable the identification of the molecules constituting the receptors. The potent α-receptor activity of the analogue HP-aminoclonidine is an indication that the amino group of p-amino-clonidine will accept

modification by potentially reactive of fluorescent groups with retention of biological activity. This will be useful to produce a variety of α-receptor probes of high affinity.

The NG108-15 system is one of the best available systems to study α_2-receptor-adenylate cyclase interaction. It may serve as a model to study presynaptic α-receptors that regulate norepinephrine release in the sympathetic nervous system. These receptors may be involved in blood pressure regulation and in the etiology or pathogenesis of essential hypertension.

Proposed Course:

Work on this project in this laboratory has been suspended. The development of useful affinity labels for α-adrenergic receptors is being pursued by Dr. Atlas, who has returned to the Hebrew University, Jerusalem, Israel. Suitable compounds should be tested in the NG108-15 system, and this may enable a future collaboration with the NIH.

Publications:

1) Atlas, D. and Sabol, S.L.: Interaction of clonidine and clonidine analogues with α-adrenergic receptors of neuroblastoma x glioma hybrid cells and rat brain. Eur. J. Biochem. 113: 521-529 (1981).

Annual Report of the
Pulmonary Branch
National Heart, Lung, and Blood Institute
October 1, 1980 through September 30, 1981

I. Basic Studies of the Control of Synthesis and Degradation of the Extracellular Matrix. Current concepts of the extracellular matrix of the alveolar structures suggest it is composed of four general classes of materials: (1) collagen (types I and III are the interstitial collagens, types IV and V are the principle basement membrane collagens); (2) elastic fibers; (3) proteoglycans; and (4) various glycoproteins (e.g., fibronectin, laminin). In the past year the major findings in this area have been as follows:

(1) Regulation of collagen production by human fibroblasts. Human lung fibroblasts produce approximately 500,000 collagen chains/hour, 90% of which are type I collagen and 10% type III. These fibroblasts maintain rigid control over collagen production during periods of rapid and slow growth but exogenous stimuli may modulate the rate of collagen production. The three major mechanisms that fibroblasts use to regulate collagen production include changing of collagen mRNA levels, rate of translation of collagen mRNA, and intracellular degradation of newly synthesized collagen. For example, while fibroblasts produce the same amount of collagen during periods of rapid and slow growth, collagen mRNA levels are twice as high in the slowly growing phase as in the rapidly growing phase and intracellular degradation of collagen is much higher during rapid growth compared to slow growth. One of the important developments in the evaluation of fibroblast collagen production is an adaptation of the enzyme-linked immunoassay (ELISA) to quantitate connective tissue proteins. This allows rapid evaluation of large numbers of cultures and avoids the problems of changes in intracellular specific activity of precursors inherent in isotope methods. It is clear that a variety of endogenous influences can modulate collagen production, one of the most important of which is the β-adrenergic system. β-agonists "down regulate" fibroblasts collagen production, at least in a short term. The mechanism is modulated through the β-agonist induced elevation of cyclic AMP levels in fibroblasts. When this occurs, the levels and activity of collagen mRNA does not change, but intracellular degradation of collagen markedly increases and the rate of collagen mRNA utilization is reduced. When intracellular cyclic AMP is increased in the fibroblasts, the decrease in collagen production appears to be type-specific; type III collagen production remains constant but type I collagen production markedly decreases. The influence of endogenous mediators on fibroblast collagen production appears to be mediated specifically via the membrane receptors. For example, β-agonists and PGE_1 both raise cyclic AMP and reduce collagen production but appear to do so through different membrane interactions. When fibroblasts are forced to make "defective" (i.e., non-helical) collagen, intracellular degradation of newly synthesized collagen markedly increases. Several pieces of evidence have shown that at least part of this intracellular degradation involves the lysosomal system; defective collagen is "shunted" into the lysosomes where it is degraded by lysosomal proteinases. This is the likely mechanism for the decreased collagen production by fibroblasts in ascorbic acid efficiency. Ascorbic acid is an important cofactor for hydroxylation of prolyl residues in collagen, an

acid is an important cofactor for hydroxylation of prolyl residues in collagen, an important mechanism for the maintenance of the helical state. In ascorbic acid deficiency, increased amounts of non-helical collagen are made and intracellular collagen degradation markedly increases. The suppressed collagen production ascorbate deficient fibroblast can be augmented by PGE_1 suggesting that delayed wound-healing in scorbutic animals may be a combination of increased intracellular degradation of defective collagen together with increased cyclic AMP-mediated degradation further decreases in collagen production. The β-agonist suppression of collagen production through surface receptors (causing the stimulation of intracellular cyclic AMP) also provides a possible mechanism by which local inflammatory processes can lead to localized fibrosis, i.e., proteases may "strip" surface β-receptors leading to a loss of normal supression of collagen production by β-agonists. Evaluation of this hypothesis has shown that proteases such as elastase have this property suggesting that active proteases present in the alveolar structures can increase connective tissue levels by decreasing the normal suppression of collagen production by β-agonists.

(2) Regulation of collagen and elastin production in organ development. Studies of collagen production in tendon, skin, and lung of sheep of various gestational ages have demonstrated that mRNA levels significantly control the production of collagen by these tissues. However, in organs such as skin, high mRNA levels can be present but the cells may not utilize all of this mRNA. Methods have been developed to evaluate elastin production by nuchal ligament and lung. During the fetal maturation in the sheep, both organs markedly increase their levels of elastin (per unit weight). This increase is paralleled by markedly increased rates of elastin production per cell. Evaluation of the mechanism modulating this increase has demonstrated that at least a significant part appears to be controlled at the transcriptional level. Methods have been developed to evaluate elastin and mRNA levels using elastin-specific complementary DNA probes. Using both solution hybridization and filter paper hybridization methodologies, evaluation of maturing nuchal ligament and lung have demonstrated the rates of elastin production by both tissues are paralleled by markedly increased levels and activity of elastin mRNA per cell.

(3) Evaluation of collagen and elastin gene structure. Our laboratory has continued to evaluate connective tissue gene structure through the isolation of connective tissue specific-genes using recombinant DNA methodology. Studies have continued on the sheep pro α2 gene. In addition, we have now isolated a portion of the human pro α2 gene and portions of the sheep elastin gene. Large portions of these genes are composed of non-coding information ("introns") with only small portions representing structural information ("exons"). Purification of portions of sheep pro α2 genes have demonstrated this gene is represented only once in the sheep genome. In addition, sequencing of the region of the gene representing the C-terminal region of the pro α2 molecule has shown remarkable homology (greater than 85% of bases) with the chick pro α2 gene in the same region.

(4) Connective tissue production by lung cells. Evaluation of connective tissue production by cultured rat pleural mesothelial cells has demonstrated that collagen rep-

resents 3% of total protein synthesized and 30% of all secreted proteins. The cells produce collagen types I, III, and IV, elastin, fibronectin, and laminin. Thus, pleural mesothelial cells are capable of synthesizing a wide variety of connective tissue components that can interact with each other thus contributing to the integrity and mechanical properties of this specialized structure.

(5) Destruction of connective tissue components. It has been suggested that the alveolar macrophage, by virtue of its numbers and location within the alveolar structures, may participate in connective tissue remodeling by producing connective tissue specific proteases. However, evaluation of human alveolar macrophages demonstrate that while the cells are capable of de novo production of small amounts of collagenase and elastase, the release of these proteases appears to be "constitutive" i.e., there is no augmentation in response to inflammatory stimuli. Thus, production of these connective tissue specific proteases by the human alveolar macrophage may represent a vestigial biological function reflecting a high level of differentiation of this phagoctyic cell. Methods have been developed in the past year to purify eosinophils to evaluate their role in connective tissue destruction. Guinea pig and human eosinophils contain collagenase that is active against both human lung type I and type III collagens. This collagenase produce specific cleavage products and is inhibited by EDTA, consistent with the concept of a "classic" collagenase. Thus, it is likely that the eosinophil participates in the disordering of collagen accompanying diseases in which there is chronic accumulation of the eosinophils within connective tissue structures.

II. Relationship of the Inflammatory and Immune Systems to the Maintenance of Lung Structure and Function. The inflammatory and immune system is an important determinant of lung structure and function in health and disease. The Pulmonary Branch has a broad based program to evaluate the composition and function of the cellular and noncellular components of this system in animal models and in human lung. One major advance has been the development of methods to isolate inflammatory and immune effector cells and noncellular components from human lung by the technique of bronchoalveolar lavage, in which the fiberoptic bronchoscope is used to "wash out" a small segment of the epithelial surface of the lower respiratory tract. In the past year, these studies have included:

(1) Recruitment of inflammatory and immune effector cells to the lung. A number of acute and chronic lung disorders are associated with accumulation of neutrophils within the alveolar structures. Recent studies have demonstrated that the alveolar macrophage is capable of being stimulated to produce a chemotactic factor that is specific for neutrophils. Some of the stimuli that will induce alveolar macrophages to release this chemotactic factor include immune complexes, particulates, and infectious organisms. The chemotactic factor is of low molecular weight and predominantly lipid in nature. Two animal models have been utilized to demonstrate that this macrophage-derived neutrophil chemotactic factor is a mechanism by which neutrophils accumulate in the lungs. In guinea pigs following acute asbestos exposure there is a rapid accumulation of neutrophils within the alveolar structures. The same is true in a guinea pig model of acute hypersensitivity pneumonitis in which guinea pigs sensitive to ovalbumin are given intratracheal particulate

ovalbumin. In both models, recovery of alveolar macrophages demonstrate that they are spontaneously producing the neutrophil chemotactic factor. In addition, alveolar macrophages from normal animals exposed in vitro to asbestos or particulate ovalbumin are induced to produce this chemotactic factor. Other studies have demonstrated that fragments of elastin are capable of attracting monocytes to the lung parenchyma. Since a variety of human disorders are associated with destruction of elastin fibers, this gives a mechanism for the accumulation of mononuclear phagocytes within the lung parenchyma.

(2) Animal models. An animal model of beryllium lung disease has been established in which guinea pigs are exposed to BeF_2 by intradermal injections over a period of six weeks. These animals demonstrate an alveolitis characterized by increased numbers of lymphocytes and macrophages. Lung lymphocytes of intradermally exposed animals spontaneously replicate and demonstrate sensitivity to beryllium in vitro. In addition, pulmonary alveolar macrophages of guinea pigs exposed to beryllium intradermally are spontaneously producing interleukin-1 (lymphocyte activating factor) consistent with in vivo activation of these macrophages. Interestingly, these facts occur without direct lung exposure, suggesting the origins of beryllium lung disease may be found in a variety of exposure settings other than inhalation. Genetically controlled responses of the lung to endogenous agents that cause lung disease have been evaluated in animals of different genetic strains and animals of the same genetic strains with different histocompatibility loci. Using bleomycin (a chemotherapeutic agent that induces lung disease) and a model of hypersensitivity pneumonitis, these studies have demonstrated overall genetic background as well as the histocompatibility locus significantly modulate susceptibility to disease. Interestingly, although the models used are very different, the same strains of mice susceptible to hypersensitivity pneumonitis are also susceptible to drug-induced disease, suggesting that genetic background can have a general affect on disease susceptibility that is not disease-specific. Studies have continued in evaluating the "tight skin" mouse, a new hereditary animal model of emphysema. Although these animals have a skin that is similar to the human disease scleroderma, their lungs have morphologic and physiologic abnormalities similar to that of human panacinar emphysema. Though the mechanism is still unknown, it does appear that these animals have increased numbers of neutrophils in their alveolar structures suggesting an excess burden of proteases in the lung may contribute to their characteristic destructive lung disease. Studies have also continued on a new animal model of hereditary interstitial lung disease in the "moth-eaten" mouse. This animal has a number of immune abnormalities, the most striking of which are increased immunoglobulin production in the lung associated with intra-alveolar hemmorhage, phagocytosis of erythroyctes by macrophages, and accumulation of neutrophils within the alveolar structures.

(3) Oxidant injury to the lung. Methods have been developed to directly evaluate oxidant injury to the lung parenchyma of animals and humans. Pieces of lung are labeled with ^{51}Cr (a classic method for evaluating cell cytotoxicity). Small pieces of these labeled explants are then incubated with various mediators or cells thought to initiate oxidant injury. Using these methods, studies have demonstrated that paraquat (herbicide which is known to cause fatal lung damage) can

directly injure parenchymal lung cells through oxidant mechanisms. The same is true for high concentrations of oxygen, a serious problem with patients with whom oxygen must be given to support life. Furthermore, these studies suggest that bleomycin (a chemotherapeutic agent) can directly induce lung cell injury and such injury is augmented by hyperoxia. Neutrophils can directly injure parenchymal lung cells and oxidants released by neutrophils are involved in this process. The ^{51}Cr labeled explant method has been utilized to evaluate radiation pneumonitis in which injury to the lung cell by radiation can be quantitated and various agents in protecting the lung can be evaluated. Studies are also ongoing to evaluate the role of the eosinophil in injuring lung parenchymal cells. When eosinophils are placed on cultured lung mesothelial cells, epithelial cells, or fibroblasts, injury to these cells can be demonstrated. The most sensitive cell appears to be epithelial cells, consistent with it being that cell that is first injured in eosinophil induced lung parenchymal disease.

(4) Growth factors. One mechanism to explain the fibrosis of the interstitial lung diseases is that the collagen producing cells of the lung are increased in number even though they may be producing the same number of collagen molecules per cell. To evaluate the hypothesis that the alveolar macrophage may produce a growth factor that induces human fibroblasts to replicate, a variety of stimuli have been used to activate alveolar macrophages in humans. Supernatants from these macrophages have been added to human lung fibroblasts in culture and the number of cells counted as a function of time. A growth factor has been identified for both animal and human alveolar macrophages. In addition, particulates such as asbestos will induce the alveolar macrophage to produce this growth factor thus explaining one of the mechanisms of fibrosis in this disease. Detailed study of the alveolar macrophage derived growth factor has demonstrated that it is different than all other described growth factors. It acts in the cell cycle as a "progression" factor, i.e., it has a similar mode of action to the somatomedins. Furthermore, evaluation of various human lung fibroblast strains demonstrated that fibroblasts vary several fold in their response to this growth factor.

III. Clinical Studies of Lung Disease. The clinical studies of the Pulmonary Branch involve three general categories of chronic lung disorders: interstitial lung disease, destructive lung disease, and hereditary lung disease.

(1) Interstitial lung disorders. These disorders represent 15-30% of the non-infectious disorders of the lung. In the past year, the Pulmonary Branch has continued its detailed studies of patients with interstitial disease with particular emphasis on those with idiopathic pulmonary fibrosis (IPF) and sarcoidosis.

IPF is a disorder characterized by chronic accumulation of neutrophils in the lung. Detailed studies using the methods of bronchoalveolar lavage have demonstrated that these neutrophils accumulate via a local production of immune complexes. Lung B-lymphocytes (but not not blood B-lymphocytes) are producing large amounts of immunoglobulin, some of which is directed against connective tissue components. These immunoglobulins form immune complexes within the lung. The immune complexes interact with the alveolar macrophage through Fc-receptors

resulting in the stimulation of the alveolar macrophage and resultant production of a variety of factors critically important to the pathogenesis of this disease. One of these factors is the chemotactic factor for neutrophils that provides a local chemoattractant gradient that attracts neutrophils to the alveolar structures from pulmonary capillaries. The neutrophil carries a wide armamentarium of mediators that induce much of the derangement that is characteristic of this disease. Studies of the relevance of the accumulation of neutrophils in the alveolar structures in IPF has demonstrated that those individuals with high intensity neutrophil alveolitis (neutrophils >10% of all lung inflammatory and immune effector cells) deteriorate significantly faster than those individuals with low intensity neutrophil alveolitis (neutrophils comprising ≤10% all lung effector cells). In this context, a randomized protocol has been carried out in which large doses of parenteral cortiosteroids have been used in a "pulse" fashion to suppress the neutrophil alveolitis of IPF. Evaluation of these patients by bronchoalveolar lavage and by gallium-67 scanning has shown a significant suppression in those individuals receiving 2 gm solu-medrol on a weekly basis on a background of a low dose of oral corticosteroids therapy.

Evaluation of patients with pulmonary sarcoidosis using bronchoalveolar lavage have led to major insights into the pathogenesis of this disorder. Not only is pulmonary sarcoidosis characterized by increased proportions of T-lymphocytes in the alveolar structures but these T-lymphocytes are represented by a shift in subpopulations such that there is markedly increased helper-T cells and reduced suppressor-T cells. Interestingly, this phenomenon occurs only at sites of disease activity such as the alveolar structures; e.g., it is not observed in studies of blood. Correlation of function with these subpopulations of lung T-lymphocytes has shown that in active sarcoidosis not only are increased proportions of helper-T cells, but the T-cell populations are producing monocyte chemotactic factor and are producing "helper" factors that nonspecifically stimulate lung B-lymphocytes in a polyclonal fashion to produce immunoglobulins. The latter mechanism likely explains why patients with sarcoidosis have large amounts of circulating immunoglobulins directed against a variety of antigens, i.e., these immunoglobulins are produced at sites of disease activity and diffuse into the blood. Although the relevance of this hypergammglobulinemia to the pathogenesis of sarcoidosis is unknown, it likely is an epiphenomon related to the lung T-lymphocyte activation. Evaluation of alveolar macrophages in these patients has demonstrated that they are producing large amounts of interleukin-1 (also called "lymphocyte activating factor"), a product which likely expands the numbers of T-lymphocytes within the alveolar structures. Thus, the alveolitis of pulmonary sarcoidosis is characterized by activated T-lymphocytes and an activation of at least one population of alveolar macrophages. Two methods have been used to stage pulmonary sarcoidosis: bronchoalveolar lavage quantitation of lung-T lymphocytes and gallium-67 scanning. If the sarcoid patient has high intensity alveolitis (>28% T-lymphocytes plus a positive gallium scan), the large majority will deteriorate in at least one functional parameter over the subsequent six months. In contrast, if the individual has low intensity alveolitis (T-lymphocytes <28% and/or a negative gallium scan) the vast majority will stabilize or improve over the same time period. It has been suggested that serum angiotensin converting enzyme is one method by

which to stage patients with pulmonary sarcoidosis. However, direct comparison of serum angiotensin converting enzyme levels with bronchoalveolar lavage and gallium-67 scanning assessment of the alveolitis of sarcoidosis has shown little correlation, i.e., serum angiotensin converting enzyme is less sensitive to the alveolitis of sarcoidosis than methods such as lavage and gallium scanning. In addition, evaluation of serum angiotensin converting enzyme in bronchoalveolar lavage fluid of sarcoid patients shows it increased many fold over that in blood. However, that increase is not disease-specific; i.e., it is found in other interstitial lung diseases as well as in normals. Bronchoalveolar lavage and gallium-67 assessment of the alveolitis and sarcoidosis has provided the clinician with a new approach to making therapy decisions. Since patients with high intensity alveolitis deteriorate, prospective studies are being used to evaluate the effects of corticosteroids in these patients. The results have been striking; in contrast to untreated patients, those patients with high intensity alveolitis that are treated with corticosteroids either stabilize or improve. Furthermore, a new approach to therapy of pulmonary sarcoidosis has been undertaken by the Pulmonary Branch utilizing short course intraveneous "pulse" therapy with large doses of corticosteroids. Using a once per week schedule for six weeks, 2 gm solu-medrol appears to significantly suppress the alveolitis in at least 70% of those tested. Interestingly, even though the therapy is only continued for six weeks, this suppression of the alveolitis persists for at least six months. This may provide the clinician with an approach to circumvent the chronic use of corticosteroids (with less intense side effects) and to adequately suppress disease activity and prevent deterioration by intermittent therapy.

Fibronectin is a large glycoprotein that mediates the attachment of cells to the connective tissue matrix. Evaluation of bronchoalveolar lavage fluid from patients with interstitial lung disease has demonstrated increased amounts of fibronectin in the lower respiratory tract in these patients. Furthermore, evaluation of alveolar macrophages from these patients shows these cells produce fibronectin, and that macrophages from patients with interstitial lung disease are producing much larger amounts of fibronectin than macrophages of normals. This fibronectin is chemotactic for lung fibroblasts thus providing a mechanism for the recruitment of fibroblasts to sites of tissue injury, i.e., macrophages are activated, produce fibronectin, and this fibronectin recruits fibroblasts and subsequently attaches them to the extracellular matrix. In addition to producing fibronectin, alveolar macrophages from patients with active interstitial lung disease are also producing the macrophage-derived growth factor for human lung fibroblasts. This growth factor stimulates lung fibroblasts to replicate. Thus, the macrophage not only attracts fibroblasts but locally expands their numbers by secreting a growth factor into the local milieu. Evaluation of inflammatory and immune effector cells in the lower respiratory tract patients with interstitial lung disease has shown that these cells mediate the positive thoracic gallium scans in these patients. Bronchoalveolar lavage of individuals receiving gallium has shown that greater than 99% of the gallium is localized to the effector cells. In addition, the primary cell localizing gallium is the activated alveolar macrophage. In idiopathic pulmonary fibrosis, the neutrophil also contributes to gallium accumulation in the alveolar structures.

Prospective evaluation of the safety of fiberoptic bronchoalveolar lavage in evaluation of interstitial lung diseases has shown that it is a remarkably safe procedure; less than 5% of patients have any complications and all of the complications are minor and easily treated. Although physiologic methods are not very specific or sensitive to the inflammatory process they do provide information concerning the derangements to the alveolar structure that have occurred in interstitial disease. Evaluation of inspiratory muscle force in severe interstitial lung disease has shown that it is normal. Thus, these individuals do not have excess "training" of inspiratory muscles even though their lungs are much "stiffer" than normal. Detailed morphologic studies at the ultrastructural level of patients with interstitial lung disease have continued. It has been recognized for some time that epithelial cells of the alveolar structures of these patients are markedly altered. Ultrastructural studies have shown that the epithelial changes include at least three cell types: normal type II epithelial cells and two types of bronchiolar cells (cuboidal cells A and B). In addition, patients with interstitial lung disease also have significant numbers of Langerhan's cells in the alveolar structures. While these are most prominent in diseases like histiocytosis-X, these mononuclear phagocyte series-derived cells are found in a variety of lung diseases. However, these cells are present in bronchoalveolar lavage fluid of only patients with histiocytosis-X thus providing an ultrastructural "marker" that seems to be diagnostic for this disease.

(2) Pathogenesis and therapy of destructive lung disease. The destructive lung disorders are disorders in which the alveolar structures are lost. The current concepts of the pathogenesis of these disorders are defined by the so-called "protease-antiprotease" theory of emphysema. This theory holds that in the normal lung there is a balance between the proteases (e.g., elastase) released by inflammatory and immune effector cells within the lung, balanced by antiproteases (e.g., α1-antitrypsin, a circulating antiprotease that diffuses into the lung). In the destructive lung diseases, presumably there is an imbalance such that the proteases overpower the antiprotease systems, thus causing connective tissue destruction and loss of the alveolar structures. The interest of the Pulmonary Branch in the destructive lung diseases has been directed toward evaluating the influence of smoking on destruction in the lower respiratory tract and evaluation of the pathogenesis and therapy of α1-antitrypsin deficiency, a hereditary disorder associated with early onset panacinar emphysema. Alpha 1-antitrypsin is the major antielastase of the lower respiratory tract. In cigarette smokers α1-antitrypsin levels in lung are normal. However, functional evaluation of the α1-antitrypsin in the alveolar structures of cigarette smokers has shown that it is at least 50% functionally deficient (i.e., it is present but not working properly). The likely mechanism for this is that cigarette smoke, either by itself or through inflammatory and immune effector cells, oxidize a methionine group near the active site of this antiprotease. Direct evaluation of lung biopsies of smokers has shown that they have neutrophils within the lung. Since these cells carry elastase and other proteolytic enzymes, this is likely a critical mechanism for the destruction of lung in this disease. A detailed evaluation has been carried out of the protease-antiprotease balance within the lower respiratory tract of normal individuals and individuals with α1-antitrypsin deficiency. Whereas α1-antitrypsin is the major antielastase of

normal individuals, individuals with a deficiency of this enzyme in the serum have no antielastase protection in the lower respiratory tract. Furthermore, recent studies have shown that patients with α1-antitrypsin deficiency had neutrophils within their lungs and active neutrophil elastase can be recovered from bronchoalveolar lavage fluid derived from the lower respiratory tract. While the mechanism of the neutrophil accumulation in the alveolar structures is not known, evaluation of the alveolar macrophages of these patients shows that significant numbers are spontaneously producing the chemotactic factor for neutrophils. Two therapeutic protocols have been carried out for destructive lung disease, both involving patients with homozygous PiZ α1-antitrypsin deficiency. The impeded androgen Danacol has been used for one month period in a group of patients; in the vast majority, α1-antitrypsin levels increased approximately 50%. While this does not cure the disease, it does bring the α1-antitrypsin levels closer to the theoretical protective threshold (70 mg/dl) of serum α1-antitrypsin. A direct therapeutic approach to α1-antitrypsin deficiency has been taken in which 5 patients were treated with intravenous replacement of α1-antitrypsin derived from pooled plasma of normal individuals. Each individual was given 4 gm of α1-antitrypsin intravenously over an 8 hour period once a week for 4 weeks. In all 5, the levels achieved for the one month period were consistently above 80 mg/dl. In addition, direct evaluation of the antiprotease screen of the lower respiratory tract of these patients has shown that whereas they were devoid of antielastase protection before therapy, after intravenous α1-antitrypsin infusions, they had adequate lung antielastase protection. Furthermore, following the intraveneous α1-antitrypsin infusions, the active elastase present in their alveolar structures is completely suppressed. It appears, therefore, that this specific therapy is a rational approach to the prevention of the destruction of the alveolar structures of this generally fatal disorder.

(3) Hereditary lung disease. Familial pulmonary fibrosis is a disease identical to idiopathic pulmonary fibrosis except that it occurs as an autosomal dominant trait with incomplete penetrance. Evaluation of these families provides a remarkable opportunity to study the pathogenesis of this disease. In this regard, one family has been evaluated in detail. In the first generation, several individuals had biopsy proven familial pulmonary fibrosis. These individuals also had all the hallmarks of this disease present in bronchoalveolar lavage evaluation; the presence of neutrophils, the activation of alveolar macrophages to produce neutrophil chemotactic factor, production of growth factor by alveolar macrophages, and the increased production of any immunoglobulins by lung B-lymphocytes. Remarkably, evaluation of the second generation (none of whom has any clinical, x-ray, or physiologic evidence of disease) has shown that several members have these inflammatory and immune stigmata of the disease in their respiratory tract. From what is understood of the pathogenesis of this disease, this data strongly suggests the alveolitis preceeds the derangements of the alveolar structures that causes loss of lung function that is eventually fatal to these patients. The family members are being closely evaluated on a yearly basis to follow the natural history of this disorder.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02405-08 PB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

Relationship of Inflammatory and Immune Systems to the Maintenance of Lung Structure and Function

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 inflammatory and immune system is critical to the maintenance of lung structure and function. It does so by recruiting blood inflammatory and immune effector cells to the lung by production of chemotactic factors. Animal models have been established for beryllium lung disease. Studies of various genetic strains of animals have shown that the same strains of animals are sensitive to bleomycin induced lung disease and hypersensitivity pneumonitis. A new animal model of interstitial lung disease (the moth-eaten mouse) has been established as well as a new animal model in emphysema (the tight skin mouse). Evaluation of oxidant injury to the lung has shown that this is likely an important mechanism in lung damage induced by paraquat, high concentrations of oxygen, neutrophils, radiation, and bleomycin. Alveolar macrophages have been evaluated for the production of growth factors that induce fibroblasts to replicate. This growth factor has been characterized and its effect on lung fibroblasts detailed.

PHS-6040
(Rev. 2-81)

Other:	P. Broska	Bio Lab Tech	NHLBI PB
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Project Description:

Objectives: The vast number of chronic disorders of the alveolar structures are associated with an alveolitis, i.e., the accumulation of inflammatory and immune effector cells within the alveolar interstitium and alveolar air spaces. In the past year the Pulmonary Branch has continued a broad based program to evaluate the composition and effector function of the alveolitis of these disorders. Our objectives in the past year have been to: (1) evaluate the mechanisms of inflammatory and immune effector cell traffic into the alveolar structures; (2) develop animal models of chronic lung disease in which the influence of the inflammatory and immune systems can be evaluated; (3) evaluate how lung parenchymal cells are injured in chronic lung disease particularly as mediated by oxidants; and (4) evaluation of the role immune effector cells on the growth of lung parenchymal cells.

Major Findings: In the past year, these studies have included:

(1) Recruitment of inflammatory and immune effector cells to the lung. A number of acute and chronic lung disorders are associated with accumulation of neutrophils within the alveolar structures. Recent studies have demonstrated that the alveolar macrophage is capable of being stimulated to produce a chemotactic factor that is specific for neutrophils. Some of the stimuli that will induce alveolar macrophages to release this chemotactic factor include immune complexes, particulates, and infectious organisms. The chemotactic factor is of low molecular weight and predominantly lipid in nature. Two animal models have been utilized to demonstrate that this macrophage-derived neutrophil chemotactic factor is a mechanism by which neutrophils accumulate in the lungs. In guinea pigs following acute asbestos exposure there is a rapid accumulation of neutrophils within the alveolar structures. The same is true in a guinea pig model of acute hypersensitivity pneumonitis in which guinea pigs sensitive to ovalbumin are given intratracheal particulate ovalbumin. In both models, recovery of alveolar macrophages demonstrate that they are spontaneously producing the neutrophil chemotactic factor. In addition, alveolar macrophages from normal animals exposed in vitro to asbestos or particulate ovalbumin are induced to produce this chemotactic factor. Other studies have demonstrated that fragments of elastin are capable of attracting monocytes to the lung parenchyma. Since a variety of human disorders are associated with destruction of elastin fibers, this gives a mechanism for the accumulation of mononuclear phagocytes within the lung parenchyma.

(2) Animal models. An animal model of beryllium lung disease has been established in which guinea pigs are exposed to BeF_2 by intradermal injections over a period of six weeks. These animals demonstrate an alveolitis characterized by increased numbers of lymphocytes and macrophages. Lung lymphocytes of intradermally exposed animals spontaneously replicate and demonstrate sensitivity to beryllium in vitro. In addition, pulmonary alveolar macrophages of guinea pigs exposed to beryllium intradermally are spontaneously producing interleukin-1 (lymphocyte activating factor) consistent with in vivo activation of these macrophages. Interestingly, these facts occur without direct lung exposure, suggesting the origins of beryllium

lung disease may be found in a variety of exposure settings other than inhalation. Genetically controlled responses of the lung to endogenous agents that cause lung disease have been evaluated in animals of different genetic strains and animals of the same genetic strains with different histocompatibility loci. Using bleomycin (a chemotherapeutic agent that induces lung disease) and a model of hypersensitivity pneumonitis, these studies have demonstrated overall genetic background as well as the histocompatibility locus significantly modulate susceptibility to disease. Interestingly, although the models used are very different, the same strains of mice susceptible to hypersensitivity pneumonitis are also susceptible to drug-induced disease, suggesting that genetic background can have a general affect on disease susceptibility that is not disease-specific. Studies have continued in evaluating the "tight skin" mouse, a new hereditary animal model of emphysema. Although these animals have a skin that is similar to the human disease scleroderma, their lungs have morphologic and physiologic abnormalities similar to that of human panacinar emphysema. Though the mechanism is still unknown, it does appear that these animals have increased numbers of neutrophils in their alveolar structures suggesting an excess burden of proteases in the lung may contribute to their characteristic destructive lung disease. Studies have also continued on a new animal model of hereditary interstitial lung disease in the "moth-eaten" mouse. This animal has a number of immune abnormalities, the most striking of which are increased immunoglobulin production in the lung associated with intra-alveolar hemmorhage, phagocytosis of erythroyctes by macrophages, and accumulation of neutrophils within the alveolar structures.

(3) Oxidant injury to the lung. Methods have been developed to directly evaluate oxidant injury to the lung parenchyma of animals and humans. Pieces of lung are labeled with ^{51}Cr (a classic method for evaluating cell cytotoxicity). Small pieces of these labeled explants are then incubated with various mediators or cells thought to initiate oxidant injury. Using these methods, studies have demonstrated that paraquat (herbicide which is known to cause fatal lung damage) can directly injure parenchymal lung cells through oxidant mechanisms. The same is true for high concentrations of oxygen, a serious problem with patients with whom oxygen must be given to support life. Furthermore, these studies suggest that bleomycin (a chemotherapeutic agent) can directly induce lung cell injury and such injury is augmented by hyperoxia. Neutrophils can directly injure parenchymal lung cells and oxidants released by neutrophils are involved in this process. The ^{51}Cr labeled explant method has been utilized to evaluate radiation pneumonitis in which injury to the lung cell by radiation can be quantitated and various agents in protecting the lung can be evaluated. Studies are also ongoing to evaluate the role of the eosinophil in injuring lung parenchymal cells. When eosinophils are placed on cultured lung mesothelial cells, epithelial cells, or fibroblasts, injury to these cells can be demonstrated. The most sensitive cell appears to be epithelial cells, consistent with it being that cell that is first injured in eosinophil induced lung parenchymal disease.

(4) Growth factors. One mechanism to explain the fibrosis of the interstitial lung diseases is that the collagen producing cells of the lung are increased in number even though they may be producing the same number of collagen molecules

per cell. To evaluate the hypothesis that the alveolar macrophage may produce a growth factor that induces human fibroblasts to replicate, a variety of stimuli have been used to activate alveolar macrophages in humans. Supernatants from these macrophages have been added to human lung fibroblasts in culture and the number of cells counted as a function of time. A growth factor has been identified for both animal and human alveolar macrophages. In addition, particulates such as asbestos will induce the alveolar macrophage to produce this growth factor thus explaining one of the mechanisms of fibrosis in this disease. Detailed study of the alveolar macrophage derived growth factor has demonstrated that it is different than all other described growth factors. It acts in the cell cycle as a "progression" factor, i.e., it has a similar mode of action to the somatomedins. Furthermore, evaluation of various human lung fibroblast strains demonstrated that fibroblasts vary several fold in their response to this growth factor.

Significance to Biomedical Research and the Program of the Institute: The hypothesis that the inflammatory and immune systems are important factors in modulating the development of interstitial lung disease is becoming a proven reality. It is likely that if it is possible to effectively treat these disorders, the most vulnerable component in the pathogenesis will be at this step. A definition of the cells comprising the alveolitis of these disorders as well as the function of the cells comprising it, are critical steps in understanding the process involved in the production of interstitial lung disease.

Proposed Course: Studies will continue to utilize animal models as well as cells obtained from humans, to evaluate the role of the inflammatory and immune systems in the maintenance of alveolar structure in health and disease.

Publications:

Hunninghake, G.W., Davidson, J., Rennard, S., Szapiel, S., Gadek, J.E., and Crystal, R.G.: Mechanisms of pulmonary emphysema: Attraction of macrophage precursors to sites of disease activity by elastin fragments. Science 212, 925-927, 1981.

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Rossi, G.A., Hunninghake, G.W., Szapiel, S.V., Gadek, J.E., Fulmer, J.D., Kawanami, O., Ferrans, V.J., and Crystal, R.G.: The tight-skin mouse: An animal model of inherited emphysema. Bull. Europ. Physiopath. Resp. 16 (Suppl), 157-166, 1980.

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Crystal, R.G., and Hunninghake, G.W.: Inflammatory and Immune Processes in the normal human lung. In: The Proceedings of the 6th Irwin Strasburger Memorial Seminar on Immunology: Gregory W. Siskind, ed. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02407-07 PB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Clinical Studies of 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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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
The interstitial lung disorders represent 15 to 20 percent of all pulmonary disorders; in most cases these diseases cause significant disability and many are fatal. Studies of the natural history, etiology, pathogenesis, pathophysiology and therapy of these disorders have made major inroads into understanding these diseases. Most importantly is the development of the concept that the inflammatory and immune effector cells are critical determinants in the pathogenic process. Methodologies have been developed to evaluate the alveolitis of these patients and to examine its effect on the alveolar structures. Therapeutic trials are underway to evaluate the efficacy of drug programs aimed at irradicating the alveolitis of these diseases. Smoking induces macrophages to produce chemotactic factors for neutrophils. In addition, cigarette smoke reduces the ability of α1-antitrypsin to function normally. Therapeutic trials are ongoing for treatment of α1-antitrypsin deficiency using Danacol therapy to increase release of the antiprotease from the liver and a direct replacement trial of α1-antiproteinase is ongoing.

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(Rev. 2-81)

Other:

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Project Description:

Objectives: The interstitial lung disorders represent 15 to 30% of the non-infectious disorders of the lung. There are more than 130 separable disease entities associated with interstitial disease, but they can be categorized into two general groups: those of known etiology and those of unknown etiology. The interstitial lung diseases of known etiology include the occupational and environmental inhalents (inorganic dust, organic dust, gases, fumes, vapors, aerosols), drugs, poisons, radiation, and interstitial disease caused by disorders of organs other than lung. The interstitial lung diseases of unknown etiology include more than 20 different categories, the most important of which are idiopathic pulmonary fibrosis (IPF), chronic interstitial disease associated with the collagen vascular disorders, sarcoidosis, and eosinophilic granuloma. The Pulmonary Branch has undertaken a detailed study of patients with interstitial disease, particularly those with IPF, sarcoidosis and interstitial disease caused by inorganic and organic dusts. IPF has been of particular interest as it represents a chronic devastating illness 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. Although sarcoidosis is a less devastating illness for most patients with the disease, it represents a more common form of interstitial lung disease, occurring in 40/100,000 blacks in this country. Of those, 5 to 11% will eventually die of the disease and up to 30% are disabled by pulmonary insufficiency. The objectives of our studies are to define the natural history, etiology, pathogenesis, pathophysiology, and therapy of the interstitial lung diseases. In the past year, we have concentrated primarily on IPF and sarcoidosis.

The destructive lung disorders are disorders in which the alveolar structures are lost. Current concepts of the pathogenesis of these disorders is defined by the so-called "protease-antiprotease" theory of emphysema. This theory holds that in the normal lung there is a balance between proteases (i.e., elastase) released by inflammatory and immune effector cells both within, and migrating to the lung, balanced by anti-proteases (i.e., α1-antiproteinase, a circulating antiprotease that diffuses into the lung). In the destructive lung diseases, presumably there is an imbalance such that the proteases overpower the anti-protease systems, thus causing connective tissue destruction and loss of portions of the alveolar structures. During the past year, the interest of the Pulmonary Branch in the destructive lung disorders has been directed toward the influence of smoking on destruction in the lower respiratory tract, evaluation of the therapy of α1-antitrypsin deficiency (a hereditary disorder associated with panacinar emphysema), and various other clinical studies of lung diseases associated with destruction of the alveolar structures.

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 in-

cluding lung volumes, flow rates, diffusing capacity, flow-volume curves, closing volume, closing capacity, body plethysmography for 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 as well as non-cellular constituents of the lower respiratory tract, ventilation and perfusion scans, and gallium 67 scans. In some patients, the pulmonary circulation is evaluated with right heart catheterization at rest and exercise. When indicated lung biopsy is done either through the fiberoptic bronchoscope or via open thoracotomy. Tissues are studied by light microscopy, electron microscopy, culture, evaluation of the cellular components of the inflammatory and immune systems and evaluation of the constituents of the extracellular matrix. Selected patients are entered into drug treatment protocols aimed at halting the alveolitis of the disease.

Major Findings:

(1) Interstitial lung disorders. These disorders represent 15-30% of the non-infectious disorders of the lung. In the past year, the Pulmonary Branch has continued its detailed studies of patients with interstitial disease with particular emphasis on those with idiopathic pulmonary fibrosis (IPF) and sarcoidosis.

IPF is a disorder characterized by chronic accumulation of neutrophils in the lung. Detailed studies using the methods of bronchoalveolar lavage have demonstrated that these neutrophils accumulate via a local production of immune complexes. Lung B-lymphocytes (but not not blood B-lymphocytes) are producing large amounts of immunoglobulin, some of which is directed against connective tissue components. These immunoglobulins form immune complexes within the lung. The immune complexes interact with the alveolar macrophage through Fc-receptors resulting in the stimulation of the alveolar macrophage and resultant production of a variety of factors critically important to the pathogenesis of this disease. One of these factors is the chemotactic factor for neutrophils that provides a local chemoattractant gradient that attracts neutrophils to the alveolar structures from pulmonary capillaries. The neutrophil carries a wide armamentarium of mediators that induce much of the derangement that is characteristic of this disease. Studies of the relevance of the accumulation of neutrophils in the alveolar structures in IPF has demonstrated that those individuals with high intensity neutrophil alveolitis (neutrophils >10% of all lung inflammatory and immune effector cells) deteriorate significantly faster than those individuals with low intensity neutrophil alveolitis (neutrophils comprising ≤10% all lung effector cells). In this context, a randomized protocol has been carried out in which large doses of parenteral cortiosteroids have been used in a "pulse" fashion to suppress the neutrophil alveolitis of IPF. Evaluation of these patients by bronchoalveolar lavage and by gallium-67 scanning has shown a significant suppression in those individuals receiving 2 gm solu-medrol on a weekly basis on a background of a low dose of oral corticosteroids therapy.

Evaluation of patients with pulmonary sarcoidosis using bronchoalveolar lavage

have led to major insights into the pathogenesis of this disorder. Not only is pulmonary sarcoidosis characterized by increased proportions of T-lymphocytes in the alveolar structures but these T-lymphocytes are represented by a shift in subpopulations such that there is markedly increased helper-T cells and reduced suppressor-T cells. Interestingly, this phenomenon occurs only at sites of disease activity such as the alveolar structures; e.g., it is not observed in studies of blood. Correlation of function with these subpopulations of lung T-lymphocytes has shown that in active sarcoidosis not only are increased proportions of helper-T cells, but the T-cell populations are producing monocyte chemotactic factor and are producing "helper" factors that nonspecifically stimulate lung B-lymphocytes in a polyclonal fashion to produce immunoglobulins. The latter mechanism likely explains why patients with sarcoidosis have large amounts of circulating immunoglobulins directed against a variety of antigens, i.e., these immunoglobulins are produced at sites of disease activity and diffuse into the blood. Although the relevance of this hypergammglobulinemia to the pathogenesis of sarcoidosis is unknown, it likely is an epiphenomon related to the lung T-lymphocyte activation. Evaluation of alveolar macrophages in these patients has demonstrated that they are producing large amounts of interleukin-1 (also called "lymphocyte activating factor"), a product which likely expands the numbers of T-lymphocytes within the alveolar structures. Thus, the alveolitis of pulmonary sarcoidosis is characterized by activated T-lymphocytes and an activation of at least one population of alveolar macrophages. Two methods have been used to stage pulmonary sarcoidosis: bronchoalveolar lavage quantitation of lung-T lymphocytes and gallium-67 scanning. If the sarcoid patient has high intensity alveolitis ($>28\%$ T-lymphocytes plus a positive gallium scan), the large majority will deteriorate in at least one functional parameter over the subsequent six months. In contrast, if the individual has low intensity alveolitis (T-lymphocytes $\leq 28\%$ and/or a negative gallium scan) the vast majority will stabilize or improve over the same time period. It has been suggested that serum angiotensin converting enzyme is one method by which to stage patients with pulmonary sarcoidosis. However, direct comparison of serum angiotensin converting enzyme levels with bronchoalveolar lavage and gallium-67 scanning assessment of the alveolitis of sarcoidosis has shown little correlation, i.e., serum angiotensin converting enzyme is less sensitive to the alveolitis of sarcoidosis than methods such as lavage and gallium scanning. In addition, evaluation of serum angiotensin converting enzyme in bronchoalveolar lavage fluid of sarcoid patients shows it increased many fold over that in blood. However, that increase is not disease-specific; i.e., it is found in other interstitial lung diseases as well as in normals. Bronchoalveolar lavage and gallium-67 assessment of the alveolitis and sarcoidosis has provided the clinician with a new approach to making therapy decisions. Since patients with high intensity alveolitis deteriorate, prospective studies are being used to evaluate the effects of corticosteroids in these patients. The results have been striking; in contrast to untreated patients, those patients with high intensity alveolitis that are treated with corticosteroids either stabilize or improve. Furthermore, a new approach to therapy of pulmonary sarcoidosis has been undertaken by the Pulmonary Branch utilizing short course intraveneous "pulse" therapy with large doses of corticosteroids. Using a once per week schedule for six weeks, 2 gm solu-medrol appears to significantly suppress the alveolitis in at least 70% of those tested. Interest-

ingly, even though the therapy is only continued for six weeks, this suppression of the alveolitis persists for at least six months. This may provide the clinician with an approach to circumvent the chronic use of corticosteroids (with less intense side effects) and to adequately suppress disease activity and prevent deterioration by intermittent therapy.

Fibronectin is a large glycoprotein that mediates the attachment of cells to the connective tissue matrix. Evaluation of bronchoalveolar lavage fluid from patients with interstitial lung disease has demonstrated increased amounts of fibronectin in the lower respiratory tract in these patients. Furthermore, evaluation of alveolar macrophages from these patients shows these cells produce fibronectin, and that macrophages from patients with interstitial lung disease are producing much larger amounts of fibronectin than macrophages of normals. This fibronectin is chemotactic for lung fibroblasts thus providing a mechanism for the recruitment of fibroblasts to sites of tissue injury, i.e., macrophages are activated, produce fibronectin, and this fibronectin recruits fibroblasts and subsequently attaches them to the extracellular matrix. In addition to producing fibronectin, alveolar macrophages from patients with active interstitial lung disease are also producing the macrophage-derived growth factor for human lung fibroblasts. This growth factor stimulates lung fibroblasts to replicate. Thus, the macrophage not only attracts fibroblasts but locally expands their numbers by secreting a growth factor into the local milieu. Evaluation of inflammatory and immune effector cells in the lower respiratory tract patients with interstitial lung disease has shown that these cells mediate the positive thoracic gallium scans in these patients. Bronchoalveolar lavage of individuals receiving gallium has shown that greater than 99% of the gallium is localized to the effector cells. In addition, the primary cell localizing gallium is the activated alveolar macrophage. In idiopathic pulmonary fibrosis, the neutrophil also contributes to gallium accumulation in the alveolar structures.

Prospective evaluation of the safety of fiberoptic bronchoalveolar lavage in evaluation of interstitial lung diseases has shown that it is a remarkably safe procedure; less than 5% of patients have any complications and all of the complications are minor and easily treated. Although physiologic methods are not very specific or sensitive to the inflammatory process they do provide information concerning the derangements to the alveolar structure that have occurred in interstitial disease. Evaluation of inspiratory muscle force in severe interstitial lung disease has shown that it is normal. Thus, these individuals do not have excess "training" of inspiratory muscles even though their lungs are much "stiffer" than normal. Detailed morphologic studies at the ultrastructural level of patients with interstitial lung disease have continued. It has been recognized for some time that epithelial cells of the alveolar structures of these patients are markedly altered. Ultrastructural studies have shown that the epithelial changes include at least three cell types: normal type II epithelial cells and two types of bronchiolar cells (cuboidal cells A and B). In addition, patients with interstitial lung disease also have significant numbers of Langerhan's cells in the alveolar structures. While these are most prominent in diseases like histiocytosis-X, these mononuclear phagocyte series-

derived cells are found in a variety of lung diseases. However, these cells are present in bronchoalveolar lavage fluid of only patients with histiocytosis-X thus providing an ultrastructural "marker" that seems to be diagnostic for this disease.

(2) Pathogenesis and therapy of destructive lung disease. The destructive lung disorders are disorders in which the alveolar structures are lost. The current concepts of the pathogenesis of these disorders are defined by the so-called "protease-antiprotease" theory of emphysema. This theory holds that in the normal lung there is a balance between the proteases (e.g., elastase) released by inflammatory and immune effector cells within the lung, balanced by antiproteases (e.g., α1-antitrypsin, a circulating antiprotease that diffuses into the lung). In the destructive lung diseases, presumably there is an imbalance such that the proteases overpower the antiprotease systems, thus causing connective tissue destruction and loss of the alveolar structures. The interest of the Pulmonary Branch in the destructive lung diseases has been directed toward evaluating the influence of smoking on destruction in the lower respiratory tract and evaluation of the pathogenesis and therapy of α1-antitrypsin deficiency, a hereditary disorder associated with early onset panacinar emphysema. Alpha 1-antitrypsin is the major antielastase of the lower respiratory tract. In cigarette smokers α1-antitrypsin levels in lung are normal. However, functional evaluation of the α1-antitrypsin in the alveolar structures of cigarette smokers has shown that it is at least 50% functionally deficient (i.e., it is present but not working properly). The likely mechanism for this is that cigarette smoke, either by itself or through inflammatory and immune effector cells, oxidize a methionine group near the active site of this antiprotease. Direct evaluation of lung biopsies of smokers has shown that they have neutrophils within the lung. Since these cells carry elastase and other proteolytic enzymes, this is likely a critical mechanism for the destruction of lung in this disease. A detailed evaluation has been carried out of the protease-antiprotease balance within the lower respiratory tract of normal individuals and individuals with α1-antitrypsin deficiency. Whereas α1-antitrypsin is the major antielastase of normal individuals, individuals with a deficiency of this enzyme in the serum have no antielastase protection in the lower respiratory tract. Furthermore, recent studies have shown that patients with α1-antitrypsin deficiency had neutrophils within their lungs and active neutrophil elastase can be recovered from bronchoalveolar lavage fluid derived from the lower respiratory tract. While the mechanism of the neutrophil accumulation in the alveolar structures is not known, evaluation of the alveolar macrophages of these patients shows that significant numbers are spontaneously producing the chemotactic factor for neutrophils. Two therapeutic protocols have been carried out for destructive lung disease, both involving patients with homozygous PiZ α1-antitrypsin deficiency. The impeded androgen Danacol has been used for one month period in a group of patients; in the vast majority, α1-antitrypsin levels increased approximately 50%. While this does not cure the disease, it does bring the α1-antitrypsin levels closer to the theoretical protective threshold (70 mg/dl) of serum α1-antitrypsin. A direct therapeutic approach to α1-antitrypsin deficiency has been taken in which 5 patients were treated with intravenous replacement of α1-antitrypsin derived from pooled plasma of normal individuals. Each individual was given 4 gm of α1-antitrypsin intravenously over an 8 hour period

once a week for 4 weeks. In all 5, the levels achieved for the one month period were consistently above 80 mg/dl. In addition, direct evaluation of the anti-protease screen of the lower respiratory tract of these patients has shown that whereas they were devoid of antielastase protection before therapy, after intravenous α1-antitrypsin infusions, they had adequate lung antielastase protection. Furthermore, following the intraveneous α1-antitrypsin infusions, the active elastase present in their alveolar structures is completely suppressed. It appears, therefore, that this specific therapy is a rational approach to the prevention of the destruction of the alveolar structures of this generally fatal disorder.

(3) Hereditary lung disease. Familial pulmonary fibrosis is a disease identical to idiopathic pulmonary fibrosis except that it occurs as an autosomal dominant trait with incomplete penetrance. Evaluation of these families provides a remarkable opportunity to study the pathogenesis of this disease. In this regard, one family has been evaluated in detail. In the first generation, several individuals had biopsy proven familial pulmonary fibrosis. These individuals also had all the hallmarks of this disease present in bronchoalveolar lavage evaluation; the presence of neutrophils, the activation of alveolar macrophages to produce neutrophil chemotactic factor, production of growth factor by alveolar macrophages, and the increased production of any immunoglobulins by lung B-lymphocytes. Remarkably, evaluation of the second generation (none of whom has any clinical, x-ray, or physiologic evidence of disease) has shown that several members have these inflammatory and immune stigmata of the disease in their respiratory tract. From what is understood of the pathogenesis of this disease, this data strongly suggests the alveolitis preceeds the derangements of the alveolar structures that causes loss of lung function that is eventually fatal to these patients. The family members are being closely evaluated on a yearly basis to follow the natural history of this disorder.

Significance to Biomedical Research and the Program of the Institute: The interstitial lung disorders and destructive lung disorders are almost uniformly fatal and affect a significant proportion of the population. There has been little information on the natural history, etiology, pathogenesis, pathophysiology and therapy of these disorders. By combining studies of patients with these disorders with our basic research program concerning basic immunology as well as evaluation of the control of synthesis and degradation of the extracellular matrix, we expect to make major inroads into understanding and treating these disorders.

Proposed Course: Studies as outlined will be continued. As methods are developed in the basic laboratory, they will be applied to study the biopsy specimens from human lung. Particularly important are the studies in lung explants and tissue culture where the manipulation and control of connective tissue synthesis and degradation can be explored using various pharmacologic agents. Immunologic studies will continue to explore cell-mediated mechanisms in these disorders. As the result of pharmacologic agents become promising, they will be studied in patients where applicable.

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 innocuous drug, it may bring the serum level of α1-antitrypsin above the threshold level by which these patients are more at risk. Studies will also continue concerning the parenteral replacement of α1-antitrypsin in homozygotes with this disorder.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02409 06 PB

PERIOD COVERED
October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)
Control of Synthesis and Degradation of the 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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	J. Gadek	Staff Investigator	NHLBI PB
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
17	10	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)

Basic studies of the synthesis and degradation of extracellular matrix are concentrated on evaluation of collagen production by human fibroblasts. These cells regulate collgen production by altering collagen mRNA levels and by destroying newly synthesized collagen within the cell prior to secretion. Genes have been isolated for sheep and human collagen pro α2 chains. Alveolar macrophages and the eosinophils have been evaluated for their role in the destruction of the connective tissue matrix. While the alveolar macrophage produces collagenase and elastase, this production is constitutive and likely play little role in the pathogenesis of disease. However, the eosinophil can produce collagenase in significant quantities. Mesothelial cells have been evaluated in culture and have been shown to produce many types of connective tissue components including collagen types I, III, and IV, elastin, fibronectin and laminin.

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(Rev. 2-81)

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Objectives: Current concepts of the extracellular matrix of the alveolar structures suggest it is composed of four general classes of materials: (1) collagen, of which there are four types (types I and III are the interstitial collagens, types IV and V are the principle basement membrane collagens); (2) elastic fibers; (3) proteoglycans; and (4) a newly described class of glycoproteins involved in cell-cell and cell-matrix interactions (e.g., fibronectin, laminin). In the past year, our studies have concentrated on the control of connective tissue production and destruction.

Major Findings: Over the past year the major findings in this area have been as follows:

(1) Regulation of collagen production by human fibroblasts. Human lung fibroblasts produce approximately 500,000 collagen chains/hour, 90% of which are type I collagen and 10% type III. These fibroblasts maintain rigid control over collagen production during periods of rapid and slow growth but exogenous stimuli may modulate the rate of collagen production. The three major mechanisms that fibroblasts use to regulate collagen production include changing of collagen mRNA levels, rate of translation of collagen mRNA, and intracellular degradation of newly synthesized collagen. For example, while fibroblasts produce the same amount of collagen during periods of rapid and slow growth, collagen mRNA levels are twice as high in the slowly growing phase as in the rapidly growing phase and intracellular degradation of collagen is much higher during rapid growth compared to slow growth. One of the important developments in the evaluation of fibroblast collagen production is an adaptation of the enzyme-linked immunoassay (ELISA) to quantitate connective tissue proteins. This allows rapid evaluation of large numbers of cultures and avoids the problems of changes in intracellular specific activity of precursors inherent in isotope methods. It is clear that a variety of endogenous influences can modulate collagen production, one of the most important of which is the β-adrenergic system. β-agonists "down regulate" fibroblasts collagen production, at least in a short term. The mechanism is modulated through the β-agonist induced elevation of cyclic AMP levels in fibroblasts. When this occurs, the levels and activity of collagen mRNA does not change, but intracellular degradation of collagen markedly increases and the rate of collagen mRNA utilization is reduced. When intracellular cyclic AMP is increased in the fibroblasts, the decrease in collagen production appears to be type-specific; type III collagen production remains constant but type I collagen production markedly decreases. The influence of endogenous mediators on fibroblast collagen production appears to be mediated specifically via the membrane receptors. For example, β-agonists and PGE_1 both raise cyclic AMP and reduce collagen production but appear to do so through different membrane interactions. When fibroblasts are forced to make "defective" (i.e., non-helical) collagen, intracellular degradation of newly synthesized collagen markedly increases. Several pieces of evidence have shown that at least part of this intracellular degradation involves the lysosomal system; defective collagen is "shunted" into the lysosomes where it is degraded by lysosomal proteinases. This is the likely mechanism for the decreased collagen production by fibroblasts in ascorbic acid efficiency. Ascorbic

important mechanism for the maintenance of the helical state. In ascorbic acid deficiency, increased amounts of non-helical collagen are made and intracellular collagen degradation markedly increases. The suppressed collagen production ascorbate deficient fibroblast can be augmented by PGE_1 suggesting that delayed wound-healing in scorbutic animals may be a combination of increased intracellular degradation of defective collagen together with increased cyclic AMP-mediated degradation further decreases in collagen production. The β-agonist suppression of collagen production through surface receptors (causing the stimulation of intracellular cyclic AMP) also provides a possible mechanism by which local inflammatory processes can lead to localized fibrosis, i.e., proteases may "strip" surface β-receptors leading to a loss of normal supression of collagen production by β-agonists. Evaluation of this hypothesis has shown that proteases such as elastase have this property suggesting that active proteases present in the alveolar structures can increase connective tissue levels by decreasing the normal suppression of collagen production by β-agonists.

(2) Regulation of collagen and elastin production in organ development. Studies of collagen production in tendon, skin, and lung of sheep of various gestational ages have demonstrated that mRNA levels significantly control the production of collagen by these tissues. However, in organs such as skin, high mRNA levels can be present but the cells may not utilize all of this mRNA. Methods have been developed to evaluate elastin production by nuchal ligament and lung. During the fetal maturation in the sheep, both organs markedly increase their levels of elastin (per unit weight). This increase is paralleled by markedly increased rates of elastin production per cell. Evaluation of the mechanism modulating this increase has demonstrated that at least a significant part appears to be controlled at the transcriptional level. Methods have been developed to evaluate elastin and mRNA levels using elastin-specific complementary DNA probes. Using both solution hybridization and filter paper hybridization methodologies, evaluation of maturing nuchal ligament and lung have demonstrated the rates of elastin production by both tissues are paralleled by markedly increased levels and activity of elastin mRNA per cell.

(3) Evaluation of collagen and elastin gene structure. Our laboratory has continued to evaluate connective tissue gene structure through the isolation of connective tissue specific-genes using recombinant DNA methodology. Studies have continued on the sheep pro α2 gene. In addition, we have now isolated a portion of the human pro α2 gene and portions of the sheep elastin gene. Large portions of these genes are composed of non-coding information ("introns") with only small portions representing structural information ("exons"). Purification of portions of sheep pro α2 genes have demonstrated this gene is represented only once in the sheep genome. In addition, sequencing of the region of the gene representing the C-terminal region of the pro α2 molecule has shown remarkable homology (greater than 85% of bases) with the chick pro α2 gene in the same region.

(4) Connective tissue production by lung cells. Evaluation of connective tissue production by cultured rat pleural mesothelial cells has demonstrated that collagen rep-

resents 3% of total protein synthesized and 30% of all secreted proteins. The cells produce collagen types I, III, and IV, elastin, fibronectin, and laminin. Thus, pleural mesothelial cells are capable of synthesizing a wide variety of connective tissue components that can interact with each other thus contributing to the integrity and mechanical properties of this specialized structure.

(5) Destruction of connective tissue components. It has been suggested that the alveolar macrophage, by virtue of its numbers and location within the alveolar structures, may participate in connective tissue remodeling by producing connective tissue specific proteases. However, evaluation of human alveolar macrophages demonstrate that while the cells are capable of de novo production of small amounts of collagenase and elastase, the release of these proteases appears to be "constitutive" i.e., there is no augmentation in response to inflammatory stimuli. Thus, production of these connective tissue specific proteases by the human alveolar macrophage may represent a vestigial biological function reflecting a high level of differentiation of this phagoctyic cell. Methods have been developed in the past year to purify eosinophils to evaluate their role in connective tissue destruction. Guinea pig and human eosinophils contain collagenase that is active against both human lung type I and type III collagens. This collagenase produce specific cleavage products and is inhibited by EDTA, consistent with the concept of a "classic" collagenase. Thus, it is likely that the eosinophil participates in the disordering of collagen accompanying diseases in which there is chronic accumulation of the eosinophils within connective tissue structures.

Significance to Biomedical Research and the Program of the Institute: The extracellular matrix of the alveolar structures is a critical determinant of lung structure and function. Mechanical properties of lung are intimately involved with the type, form, location and orientation of extracellular matrix. In addition, the topologic arrangements of cells is defined in part by the macromolecules of this matrix. It is likely that the connective tissue of the interstitium also forms a line of defense against inhaled agents and controls in part the movement of solutes and water from the capillaries to the alveolar structures. The pathogenesis of a large number of pulmonary diseases afflicting mankind are intimately involved with abnormalities in the connective tissue. The emphysematous lung disorders result from destructive processes of the extracellular matrix while the interstitial lung disorders abnormalities in the production and destruction of the collagen of the extracellular matrix. Understanding of the control of synthesis and degradation of these materials is of obvious importance in terms of understanding the pathogenesis of these disorders as well as understanding vulnerable points of attack for therapeutic intervention in these disorders.

Proposed Course: Our studies will continue to evaluate the composition and source of the macromolecules composing the extracellular matrix. In addition, detailed studies will continue on the control of the synthesis and degradation of these macromolecules at the cellular and sub-cellular levels.

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